MAMMALIAN SYNAPTIC TRANSMITTERS AND

THE PHARMACOLOGY OF CENTRAL CHOLINOCEPTIVE RECEPTORS

by

R.W. Ryall

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1964.

Owing to the complex nature of the neuropharmacological techniques employed in this investigation, the experiments in which drugs were applied electrophoretically to single neurones were carried out in collaboration with Dr. D.R. Curtis. However, I was responsible for the initiation and planning of many of the experiments described in Section V. The investigations reported in Sections IV B and IV C are entirely my own work, except for those parts which are acknowledged in 'Acknowledgements'.

.....R.W.Ryall.....

(R.W. Ryall)

As a result of the investigations in which I have participated during the tenure of my scholarship at the Australian National University, the following papers have appeared or are in course of publication.

Curtis, D.R. and Ryall, R.W. (1963). Central actions of psychotomimetics. Nature, 199, 1003-1004.

Curtis, D.R., Watkins, J.C. and Ryall, R.W. (1963). Cholinergic transmission in the central nervous system. Biochem. Pharmacol., 12, (Supplement) p. 52.

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Ryall, R.W. (1962). Subcellular distribution of pharmacologically active substances in guinea pig brain. Nature, 196, 680-681.

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Ryall, R.W., Stone, Nancy, Curtis, D.R. and Watkins, J.C. (1964). Action of acetylcholine extracted from brain on spinal Renshaw cells. Nature, 201, 1034-1035.

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I) ABBREVIATIONS

Drugs:

ACh; acetylcholine

BOL-148; bromolysergic acid diethylamide

GABA; γ-amino-n-butyric acid

DLH; DL-homocysteic acid

L-GLUT; L-glutamic acid

TEA; tetraethylammonium

Nerves:

BST; biceps semitendinosus

FDL; flexor digitorum longus

gastroc; gastrocnemius

SUP PER; superficial peroneal

Others:

C.N.S.; central nervous system

RSA; relative specific activity

TAW-buffer: triethylamine-acetic acid-water, 12:25:923 by vol.

SS-paper; Schleicher and Schüll paper no. 20436 Mgl

M; molar

mM; 10^{-3} molar

mA; 10^{-3} amp

μA; 10^{-6} amp

nA; 10^{-9} amp

II. GENERAL INTRODUCTION

Experimental evidence in support of the concept of chemical transmission of the nervous impulse from the terminations of peripheral nerves to an effector organ was first obtained early in the twentieth century. The pioneer work of Elliott (1904), Dale (1914), Dixon (1906) and Loewi (1921) (see also reviews by Dale, 1935; Loewi, 1945) implicated chemical agents in transmission from postganglionic autonomic nerves to their effector organs. In peripheral neuro-effector systems, the transmitter substances have been identified: acetylcholine (ACh) is the transmitter at postganglionic parasympathetic synapses, at all autonomic ganglionic synapses and at the skeletal neuromuscular junction (see Feldberg and Gaddum, 1934; Feldberg and Vertiainen, 1934; Dale, Feldberg and Vogt, 1936; Brown, Dale and Feldberg, 1936; Loewi, 1945), although the precise mechanism of action may be more complicated than was first envisaged (Koelle, 1962; Riker, Werner, Roberts and Kuperman, 1959; Nachmansohn, 1959; Werner and Kuperman, 1963 and Hubbard and Yokota, 1964). At most postganglionic sympathetic synapses the tranmitter is generally believed to be nor-adrenaline (see Von Euler, 1959). However, Burn and Rand (see Burn and Rand, 1962, for recent review of their hypothesis) have proposed that all postganglionic sympathetic nerves liberate ACh, which in turn liberates nor-adrenaline from a store. If the hypothesis were valid (and there are alternative interpretations of the evidence: Paton, 1963),then ACh would emerge as the sole transmitter for which there is evidence of liberation from nerve terminals in the peripheral nervous system as a direct consequence of nerve

stimulation. Although it is widely accepted that chemical transmission occurs in the mammalian central nervous system, the nature of the substances effecting transmission is far more obscure (see section IV). A number of naturally occurring substances have been proposed for the role of transmitter, but the evidence on which most of the claims are made is weak and indirect.

The first part of this thesis (section IV) represents an attempt to obtain and collate the evidence revealed by various relatively direct methods of approach. It will emerge that ACh is the only substance for which there is adequate evidence of a transmitter function. The second part of the thesis (section V) is devoted to a detailed account of the pharmacology and function of central cholinoceptive receptors. It will be shown that Renshaw cells have both nicotinic and muscarinic receptors upon the same cell and that the duration of the excitatory effect of the synaptically released acetylcholine is controlled by desensitization processes, in addition to the better known phenomena of enzymic inactivation and diffusion of transmitter away from the receptors.

III. METHODS

A) Extraction and cellular fractionation of brain homogenates

The studies which are reported in sections IV B, IV C and IV D, were carried out on homogenates of the fore-brains of rats (sections IV B, IV C and IV D), guinea pigs (section IV C) and sheep (section IV C). The brains were sectioned at the level of the tentorium cerebelli. The guinea pigs were usually killed by rapid decapitation whereas rats, and in a few experiments guinea pigs also, were anaesthetised with pentobarbitone sodium (40 mg/kg; intra-peritoneally) and then bled from the abdominal aorta. Brains were obtained from sheep immediately after death by exsanguination, or in one experiment from a sheep anaesthetised with pentobarbitone sodium, given intravenously.

The brains were removed rapidly and placed in a beaker of ice-cold sucrose solution (0.32 M), in which they were finely minced with scissors. Pooled homogenates from four to eight brains (30 brains were used for the experiments in sections IV B and IV D) were then prepared in a piston press type of homogenizer (Emanuel and Chaikoff, 1957), with a clearance of 27 μ between the rod and the annulus. The concentration of the homogenate was about 10 per cent and in some experiments the sucrose solution contained physostigmine (2.7×10^{-5}M). The homogenizer was cooled to 0-4°C by circulating ice-cold water through its outer cooling jacket. All fractionations were carried out at 0-4°C by one of two methods, as outlined in Tables 1 and 2. Method a) (Table 1) was similar to that described by De Robertis, Pellegrino de Iraldi, Rodriguez de Lorez Arnaiz and Salganicoff (1962) and Method b)(Table 2) was similar, but not identical, to that described by Whittaker (1959) and

Gray and Whittaker (1962). The primary fractions, P1, P2, P3 and S were obtained by differential centrifugation and the P2-subfractions were obtained by density-gradient fractionation. In gross appearance the fractions resembled those of the corresponding fractions described by these authors, and Fig. 1 illustrates the appearance and the position of the various fractions in the density gradients after centrifuging. However, fraction B (Method a) consisted of a fairly diffuse layer merging into fraction C, which represented a more compact layer at the junction of the 1.0 and 1.2 M sucrose solutions in the density gradient. This made consistent separation of the two layers difficult and was reflected in the variability of the relative amounts of ACh and nitrogen in the two fractions. Despite this fact, the total amount of ACh in the two fractions was remarkably constant.

For the preparation of the nuclear and cell-debris fraction (fraction P1), an MSE refrigerated centrifuge was used. Subsequent fractionations were carried out in a Spinco Model L ultracentrifuge with the number 30, 40 or SW 39 rotors. The centrifugal fields and density gradients which were used are given in Tables 1 and 2. Separation of supernatant fluids from the sediments, and of the layers of the density gradients was achieved with the aid of pasteur pipettes. The individual fractions so obtained were centrifuged to sediment the particles, which were then resuspended in 165 mM NaCl. In studies of the subcellular distribution (section IV C), the resuspended particles were heated on a boiling water bath at pH 4 for 10 min to liberate bound substances (Whittaker, 1959); supernatant fluids were similarly heated. In order to reduce the sucrose contents of the samples prior to electrophoresis

and chromatography (section III B, IV B and IV D), a further resuspension in NaCl was performed. The suspension was then recentrifuged and the particles taken up in distilled water, which was immediately acidified to pH 4, and heated to 100°C for 10 min. These procedures were found to have no effect on the ACh content of the sample.

The extracts were then treated as described in section III B.

B) Treatment and purification of extracts

Purification of extracts

Before carrying out assays for ACh, substance P, 5-hydroxytryptamine, potassium and the amino acids (section IV C), the extracts in NaCl (section III A) were centrifuged to remove the precipitated material. The supernatant fluid was adjusted to pH 7.5 and a portion used for the substance P, potassium and amino acid assays. For the ACh and 5-HT assays, the extracts were incubated with α-chymotrypsin (1 mg/ml) for 1 hr at 37°C in order to destroy substance P. After repeated application of solutions containing chymotrypsin, 5-HT was found to cause sustained contractions of the rat fundus strip, which was used for the assay of 5-HT. This difficulty was overcome by subsequently heating the chymotrypsin-treated extract at 80°C for 10 min, but care had to be taken to ensure that the pH did not exceed pH 7.5, since there was a 10 per cent destruction of ACh at pH 7.8. At pH 7.5 there was no destruction of ACh in control experiments, and there was no difference between the activity on the guinea pig ileum of such an extract and that of a similar, unincubated extract which was kept at pH 6.5 and not heated.

The experiments described in section IV B and IV D were carried

out on extracts of the crude mitochondrial (P2) fraction initially prepared as in III A, but thereafter further treated and purified as follows: the extract in distilled water (section III A) was centrifuged and the sediment was washed with water. The combined supernatant fluids (120 ml) were then freeze-dried and weighed. A portion of this crude extract was dissolved in water and carefully adjusted to pH 7 to 7.5 (glass electrode), incubated at 37°C for 1 hr with 1 mg/ml of α-chymotrypsin to destroy substance P, and heated at 80°C for 10 min to precipitate the chymotrypsin. These incubated samples were used directly for the parallel biological assays. The electrophoretic and chromatographic separations were performed on extracts which had not been treated with chymotrypsin.

Fractionation of extract from the crude mitochondrial fraction

Solvents and buffer systems

1-Butanol (A.R.) and 2-propanol (technical grade) were treated with Al/KOH and twice distilled through a column. Acetic acid (A.R.) and triethylamine (BDH, L.R. grade) were each distilled before use.

Chromatograms were run in 1-butanol-triethylamine-acetic acid-water (33:4:8:10, by vol.), the latter three components together having a pH of 4.

For paper-washing, for electrophoresis and for elution of substances from paper a triethylamine-acetic acid buffer, pH 3.9 (T.A.W. buffer) was prepared by diluting 12 ml triethylamine and 25 ml acetic acid to give 1 litre of buffer. In some experiments the triethylamine in the solvent systems was replaced by pyridine throughout the washing, electrophoretic and chromatographic procedures. Experiments in which pyridine was used will be specifically indicated in the text.

Paper for electrophoresis and chromatography

Whatman no. 3MM paper was employed for electrophoresis. Sheets of Schleicher and Schüll paper no. 2043 b. Mgl (referred to hereafter as S.S. paper) were used for chromatography.

3MM paper was always eluted for one week with 2-propanol-water (7:3, v/v) followed by acetic acid-water (1:1, v/v) for another week, and the S.S. paper was eluted for one week with water alone. These treatments were inadequate to remove biologically active substances from the paper. Hence extra eluting procedures were adopted and these followed on from the initial washing described above:-

The papers were eluted at room temperature for 10 days in 1-butanol-acetic acid-water (4:1:1, by vol.) and dried, following which they were transferred to the cold room (4°C) and washed with T.A.W. buffer for 14 days. After a final 4 days of elution with water the papers were dried. Elution with each solvent was terminated when no biological activity, as judged by contracture of the toad rectus, could be detected in the combined eluate from 4 whole sheets of paper, eluted for 20-21 hours.

S.S. paper was further washed successively with the chromatography solvent, followed by T.A.W. buffer and then water (4 days each).

High Voltage Electrophoresis

Part of the freeze-dried extract of the crude mitochondrial fraction was subjected to high voltage paper electrophoresis. The extract (610 mg;17 mg/g brain, wet weight) was dissolved completely by addition of 5.0 ml water, and 0.40 ml of this solution, or 49 mg of extract, was separated on each of seven papers. All seven electro-

phoretograms were made on the same day.

The water cooled apparatus described by Gross (1961) and modified slightly in regard to plate size by Beatty, Ennor and Magrath (1964) was employed. 3MM paper, washed as described above, and cut into sheets 29 cm x 58 cm was used, and the wicks consisted of single sheets of the same paper, 29 cm x 29 cm, wrapped in double sheets of cellophane. To obtain good contact between wicks and electrophoresis paper, sealed cylinders made from polythene film and packed with cotton wool were used, and in the electrode vessels, slits between electrode and wick compartments were packed with rolls of washed 3MM paper. In this way the possibility of contamination of buffer or paper by interfering materials diffusing from the cotton wool or electrode compartments was minimised. To avoid the possibility of contamination of papers with products of electrolysis, the buffer in the electrode vessels and the wicks were changed after each paper.

The solution to be separated by electrophoresis was applied by means of a syringe fitted with a drive-mechanism, which delivered 0.4 ml evenly in a 25 cm streak, 18 cm from the anode end of the paper. A potential gradient of 35 volts/cm was applied across the paper for 45 min in the high voltage electrophoresis apparatus. The current was fairly constant, being initially in the region of 30-34 mA, and increasing during the run to 33-36 mA, although on one occasion the current rose to 40 mA. On completion of electrophoresis, papers were dried in air for several hours, then overnight at a pressure of 1-2 mm/Hg in a specially constructed tank. The papers were stored at 4°C in an atmosphere of nitrogen until elution was carried out several days later.

Detection of electrophoretic and chromatographic patterns

Since the application of marker substances on electrophoretic or chromatographic sheets reduced the area of paper available for the separation of the extract, and might have contaminated the extract by lateral diffusion, markers were run on separate sheets, and compared with a small amount of brain extract. The positions of possible active substances in the extract were deduced by reference to the ninhydrin-positive bands. These were located by cutting off 1.25 cm strips from both sides of each electrophoretogram and chromatogram and spraying them with ninhydrin. This procedure also demonstrated the straightness of running of similar bands on different sheets.

For the detection of marker spots, the following reagents were used:- choline esters and γ-butyrobetaine methyl ester, hydroxylamine-ferric chloride spray (Whittaker and Wijesundera, 1952); acetyl-β-methylcholine, potassium bismuth tetraiodide spray (Bregoff, Roberts and Delwiche, 1953); and acetyl-DL-carnitine, iodine vapour (Brante, 1949).

Elution of bands from electrophoresis papers

Each electrophoresis paper was cut into 4 cm-wide strips parallel to the origin. These were eluted with T.A.W. buffer in the cold room (4°C) in an atmosphere of N_2 for 36 hr. Eluates from corresponding strips were pooled and freeze dried. Fractions were then stored at -15°C until required.

Chromatography of electrophoresis - fractions.

Washed S.S.-papers were cut into sheets 44.5 cm x 51 cm. The freeze-dried residue of an electrophoretic fraction was dissolved in 0.20 ml T.A.W. buffer and applied by hand across 40.5 cm at the origin.

A further 0.20 ml, used to wash out the container, was applied over the dried streak.

Descending chromatograms were run at 4°C in an atmosphere of N_2 using the chromatography solvent described above. In 21 hr the solvent front had moved about 32 cm from the origin. Papers were dried in air and then under vacuum (1-2 mmHg) for 1½ hr. Side strips were cut and sprayed with ninhydrin, after which the paper was cut into horizontal 1 or 3 cm bands and eluted for 28-30 hr in T.A.W. buffer at 4°C under N_2. The eluates from each paper were freeze-dried and stored at - 15°C until required for assay.

Biological testing of purified extracts

For testing on isolated tissues, the freeze-dried extracts were dissolved in 0.5 ml of water and small portions taken for the assays. The remainder was again freeze-dried, taken up in 0.02 ml of water, centrifuged to sediment particulate material, and then introduced into 5-barrelled micro-electrodes for testing on single neurones in the central nervous system.

C) Chemical estimation of amino acids, potassium, nitrogen and 5-hydroxytryptamine

Amino acids

The subcellular distribution of two amino acids, γ-amino-n-butyric acid (GABA) and glutamic acid, is described in section IV C. They were first separated from most of the other ninhydrin-positive components of the extracts by electrophoresis on Whatman 3MM paper in a pyridine (0.09 M) - acetic acid (0.43 M) - water buffer of pH 4: the extract was applied as a streak across the origin, and the GABA

and glutamate regions, identified by the position of marker spots applied at the edges of the paper, were eluted with water. The eluates were dried in vacuo, taken up in a small volume of water and further purified by descending chromatography in iso-propanol/water (7:3 v/v) for glutamic acid and phenol/water (4:1 v/v) for GABA. The papers were dried in air and the phenol removed with ether. They were then treated with ninhydrin, eluted with 50% ethanol, and the colour intensity of the ninhydrin-solutions estimated spectrophotometrically, as described by Porcellati and Thompson (1957), in comparison with recovered standards. Recoveries of GABA and glutamic acid were about 80 per cent.

Potassium

Potassium was estimated by flame photometry.

Nitrogen

Portions of the subcellular fractions, obtained as described in section III A were removed before acidification and heating. The nitrogen content of these samples was estimated by the Kjeldahl method.

5-Hydroxytryptamine (5-HT)

Besides assaying the 5-HT content of various brain-fractions by biological assay on the rat fundus-preparation, 5-HT was also estimated by a fluorimetric method. The brains from twenty rats were homogenized and separated by Method a) (section III A) to yield the P1, P2 and P3 fractions. The separate fractions, P2 and P3, were rapidly resuspended in water, adjusted to pH 4 and then heated at 100°C for 10 min. After cooling, the precipitates were removed by centrifuging and washed once with water. The pooled supernatant fluids were reduced in volume to about 10 ml in a rotary freeze-drying apparatus and then made up to

20 ml with water. Portions (8 ml) of these solutions were used for extraction with n-butanol and were assayed for 5-HT in an Aminco-Bowman spectrophotofluorometer, as described by Udenfriend (1962). The fluorescence in 3 N-HCl was compared with known amounts of 5-HT extracted in a similar manner. The remaining 4 ml of solution were used for biological assay on the rat fundus.

D) Biological assays

The following tissues and preparations were used to detect and assay substances present in brain extracts, and for the parallel assay of known cholinomimetic substances.

i) **Guinea pig ileum.**

Two variations in technique were employed. These were;

a) The superfused ileum (Gaddum, 1953; Adam, Hardwick and Spencer, 1954). This procedure was employed for the assay of ACh and substance P in the unpurified extracts (section IV C), and both mepyramine maleate (1 mg/l) and tryptamine hydrochloride (40 mg/l) were added to the superfusion fluid to block the effects of histamine and 5-HT respectively.

b) The ileum was suspended in a 2 ml bath. This technique was used for the assay of cholinomimetic substances in purified brain extracts and for the parallel assay of known cholinomimetic substances. In these experiments, the Tyrode solution did not contain blocking agents.

ii) Rectus abdominis muscle of the Queensland toad (Bufo marinus), suspended in Ringer's solution in a 1 ml bath.

iii) Toad atria, suspended in a 2 ml bath.

iv) The isolated perfused toad heart.

v) Rabbit or guinea pig atria, suspended in a 2 ml bath (Webb, 1950)

vi) The rat fundus (Vane, 1957), in an 8 ml bath.

vii) The blood pressure of the rat treated with neostigmine (Straughan, 1958).

viii) The blood pressure of the atropinized cat, anaesthetised with pentobarbitone sodium (40 mg/kg intraperitoneally).

ix) The semispinalis cervicis muscle of the chick (Tyler, 1960), in a 2 ml bath.

Contractions were usually recorded isotonically, but the contractions of the rat fundus and toad rectus muscles were recorded by means of spring loaded levers, similar to those described by Vane (1957). The levers operated small photo-electric transducers, and the output from these in turn actuated a pen-recorder ('Servo/Riter' - Texas Instruments Incorporated). Blood pressure was also recorded on the pen-recorder with the aid of a Statham strain-gauge-transducer (Type P23Db). Assays on the toad rectus, toad atria and rabbit and guinea pig atria were sometimes carried out in the presence of physostigmine salicylate (2.5×10^{-5} M for the toad preparations and 0.25×10^{-5} M for the rabbit and guinea pig atria).

Ringer's solutions of the following composition (in mM) were employed:

i) NaCl 111, KCl 1.8, $CaCl_2$ 1.08, $NaHCO_3$ 2.38, NaH_2PO_4 0.084, glucose 11.1. This solution was used for preparations ii, iii, and iv.

ii) NaCl 137, KCl 2.68, $CaCl_2$ 1.26, $MgCl_2$ 1.5, $NaHCO_3$ 4.3, NaH_2PO_4 0.415, glucose 5.55. This solution was used for preparations i and ix.

iii) NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.11, $NaHCO_3$ 12, NaH_2PO_4 0.42, glucose 5.5. This solution was used for bathing rabbit and guinea pig atria (preparation v).

iv) NaCl 118, KCl 4.7, $CaCl_2$ 2.51, $MgSO_4$ 1.2, $NaHCO_3$ 29.8, KH_2PO_4 1.2, glucose 10. This solution was used for the rat fundus.

Solutions were usually equilibrated with 95% O_2 + 5% C O_2, except when used to bathe heart muscle, when 100% O_2 was employed.

E) Neuropharmacological techniques

i) Micro-electrophoresis

The technique of micro-electrophoresis has recently been reviewed by Curtis (1964). Briefly, it consists of placing drug-containing solutions in micro-pipettes, of overall tip diameter 4-8 μ (the diameter of individual pipettes was about 2-3 μ), and ejecting the drugs in ionic form by the passage of appropriately directed electrical currents through the solution. In the present experiments, the pipettes were filled by centrifugation (see Curtis, 1964). The four, or occasionally six, outer barrels of the pipettes contained solutions of the drugs to be tested and the central barrel contained 4 M NaCl, which was used for recording purposes, except in the experiments in which nerve-terminals were stimulated when the central barrel was also used as a stimulating micro-electrode. Extracellular spike or field potentials were displayed on an oscilloscope after suitable amplification.

The spikes were also used to trigger a pulse generator, the output pulses of which were led to a rate-meter which in turn actuated a pen-recorder ('Recti/Riter', Texas Instruments Inc.), so that a continuous record of the frequency of firing was obtained. A detailed account of the recording and display techniques has been given by Andersen and Curtis (1964a).

ii) Studies on cortical neurones

Cats were decerebrated under fluothane anaesthesia by an intercollicular section. The cortex was then exposed in the pericruciate region by an opening of the immediately overlying bone. The dura were carefully opened with scissors, and the exposed cortex was irrigated with Ringer's solution. The surface of the cortex was stabilized by means of a small perspex pressure-plate (Phillips, 1956), through which the micropipette was inserted, after removal of a small portion of pia. Neurones were located by slowly tracking through the cortex whilst recording through the centre barrel and ejecting an excitant amino acid (DL-homocysteic acid) through an outer barrel. No attempt was made to identify the neurones, although many were presumably Betz cells.

iii) Studies on Renshaw cells

These studies were carried out on cats anaesthetized with pentobarbitone sodium (40 mg/kg intra-peritoneally). The spinal cord was exposed by a laminectomy extending from the first to the seventh lumbar segments and was severed at the lower thoracic level. The first sacral and seventh lumbar ventral roots were dissected, tied and mounted on stimulating electrodes. Renshaw cells were located and identified in the ventro-median part of the cord by their repetitive firing

in response to a single supramaximal stimulus applied to the ventral roots and by their sensitivity to ACh (Curtis and Eccles, 1958a).

iv) Studies on spinal interneurones

These neurones were located in the dorsal horn of the lower lumbar segments of the spinal cord in anaesthetised cats. They were detected by their response to the electrophoretic administration of DL-homocysteic acid and by their response to supramaximal stimulation of peripheral, usually cutaneous nerves.

v) Studies on presynaptic terminals

In these experiments the effects of electrophoretically administered drugs on the excitability of primary afferent terminals were investigated in anaesthetized cats (pentobarbitone sodium, 40 mg/kg intraperitoneally). The studies were carried out on terminals located in the dorsal or ventral horn of the lower lumbar cord. The spinal cord was sectioned in the lower thoracic region.

The centre barrel of the 5-barrelled micro-pipette was initially used to record extracellular field potentials produced by stimulation of peripheral nerves or the ventral roots. Subsequently, the centre barrel was used as a stimulating electrode (Wall, 1958; Eccles, Magni and Willis, 1962) and the impulses which were produced in the primary afferent fibres or ventral roots were recorded monophasically from the appropriate peripheral nerves or from the ventral root.

A Grass stimulator and isolation unit provided negative voltage pulses (0.2 msec duration) and was connected to the micro-pipette (usual resistance, 5-10 MΩ) with a series resistance of 10 MΩ (see Fig. 2). If the stimulus exceeded about 80 volts (current of 4-8 μA),

it was found that the field potentials subsequently recorded through the electrode were inverted, indicating extensive damage to cells in the immediate vicinity of the electrode orifice. Therefore the maximum stimulus was limited to about 60 volts, and in many experiments it was considerably less.

In order to stimulate the terminals with graded and reproducible stimuli, a resistance network was connected across the output terminals of the isolation unit (Fig. 2) and the proportion of the total voltage which could be applied was selected from this network either by means of a manually operated switch or by means of a stepping relay ('Uniselector'), which was coupled to the shutter of the Grass camera used to photograph the oscilloscope. The network was designed to cover a tenfold increase in stimulus strength, but it was sometimes adequate to use a narrower range of stimulus intensities.

IV. THE NATURE OF SYNAPTIC TRANSMITTER SUBSTANCES IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM

A) Introduction

Adrian (1924) suggested that inhibition in the central nervous system (C.N.S.) was due to the liberation and action of specific chemical substances. Later, Feldberg (quoted by Dale, 1935) obtained evidence for the liberation of ACh into the cerebrospinal fluid when the vagus nerve was stimulated, but Dale (1935) was cautious in his interpretation of this phenomenom and Feldberg and Schriever (1936) showed that the earlier results were due to the presence of physostigmine. However, it is now widely accepted that transmission across synapses in the central nervous system is brought about by chemical means (see Feldberg, 1945, 1950, 1951, 1954, 1957; Loewi, 1945; Eccles, 1953, 1957, 1961, 1964; Dale, 1954; Perry, 1956; Crossland, 1957; Paton, 1958, 1959; Curtis, Phillis and Watkins, 1961; Gaddum, 1963).

A synapse may be defined as a structure anatomically differentiated and functionally specialized for transmission between excitable cells (De Robertis, 1958; Eccles, 1964). Since pre- and postsynaptic components of the synapses in the central nervous system, as well as those of synapses at skeletal neuromuscular junctions and in autonomic ganglia, are in close apposition (see De Robertis, 1958, 1959; Eccles, 1964), the synaptic transmitters may be considered to act over relatively short distances and to be localized in their effects.

Having defined the concept of a synaptic transmitter substance, it is now necessary to set up the criteria by which they may be recog-

nized. These criteria have been enumerated by a number of investigators (Crossland, 1957; Feldberg, 1957; Paton, 1958; Curtis, 1961; Gaddum, 1962, 1963), and they are summarized below:

1) The substance must occur naturally in the C.N.S. and be stored at sites from which it is supposed to be liberated.

2) Synthesizing enzymes should be present in the presynaptic neurone.

3) Inactivating enzymes should be present to limit the duration of transmitter-effect.

4) Administration should exactly mimic the effect of the substance liberated by physiological stimulation of appropriate nervous pathways, and the site of action should be on the postsynaptic membrane.

5) The substance should be released from the presynaptic neurone in an identifiable form by appropriate excitation.

6) The pharmacological interaction between the postulated transmitter and substances which either block or enhance its effect should be identical with the interaction between these substances and the transmitter released by nervous stimulation.

Since these criteria were based mainly on evidence relating to the function of ACh as a transmitter at peripheral synapses, some of them may be superfluous when applied to central synapses. With certain reservations, criteria 1, 2, 4, 5 and 6 may be considered to be obligatory. However, if postulate 1) has been satisfied, then criterion 2 follows: if a substance is present in an appropriate locality then it must have been synthesized or transported there.

Criterion 3) may be unnecessary since either diffusion (Eccles and Jaeger, 1958) or 'binding' (Axelrod and Tomchick, 1959; Axelrod, Whitby and Hertting, 1961; Axelrod, Hertting and Potter, 1962; Green, 1962; Axelrod and Inscoe, 1963) could be more important than enzymic inactivation for the immediate removal of transmitter from the synapse.

The postulated transmitter may not necessarily act on the postsynaptic membrane. This follows from Koelle's hypothesis (Koelle, 1962) that a substance released from the presynaptic terminals may release more transmitter of the same, or possibly of a different type. Since both substances may be concerned in the transmission of specific nervous information across the synapse, both may be considered as synaptic transmitters. To decide between the two possible sites of action it would be necessary to allow all afferent connections with the neurone to degenerate. However, Koelle's views are not universally accepted (Paton, 1963; Eccles, 1964).

In the C.N.S. it may be extremely difficult to show that a substance is released in an identifiable form by adequate stimulation. When release can be demonstrated the origin is uncertain, and the release may be the consequence and not the cause of the change in neuronal activity. Moreover, the transmitter may be rapidly inactivated by enzymes, or there may be sequestration of the released transmitter by nearby tissues and this may mask any release which does occur: the observations of Brown and Gillespie (1957) and Blakely, Brown and Ferry (1963) suggest that this phenomenom occurs at adrenergic terminals in the spleen.

Thus the most important criteria to fulfil, before a substance can be accepted as a synaptic transmitter, are numbers 1), 4) and 6), with the reservation that the site of action does not have to be on the postsynaptic membrane.

A number of chemical substances have been advocated as potential synaptic transmitters in the C.N.S. Since the evidence on which these claims are based has been the subject of a number of excellent reviews (Feldberg, 1945, 1950, 1951, 1954, 1957; Perry, 1956; Crossland, 1957, 1960; Paton, 1958; Florey, 1961, 1964; Gaddum, 1963) it is not proposed to reiterate this data, except in so far as it bears on the specific problems to be discussed. However, the pharmacological evidence on which many of the claims are made is often weak (Paton, 1958). Only for the cholinergic synapses on Renshaw cells is the evidence at all strong (see section V).

In this first major section of the thesis it is proposed to present evidence concerning the nature of synaptic transmitters in the mammalian C.N.S., bearing in mind the criteria which have been enumerated. Firstly, in section IV B, evidence relating to the nature of the cholinomimetic substance present in synaptic terminals in brain will be given: it was considered that the data currently available in the literature were inadequate. Secondly (section IV C), the results of experiments on the subcellular localization of various naturally occurring substances will be discussed in relation to criterion 1. In section IV D are described the results of experiments carried out to test, by electrophoresis from a micro-electrode, the effects of electrophoretically and chromatographically purified extracts of a crude

mitochondrial extract from brain on single neurones in the feline nervous system. Then, in section IV E, the actions of several compounds, and amino acids in particular, on presynaptic terminals in the spinal cord are discussed in relation to their possible functions as the transmitters concerned in the phenomenom of presynaptic inhibition. Finally, the work of other investigators which throw light on the nature of the chemical transmitters in the mammalian C.N.S., will be briefly reviewed (section IV F). In this section particular attention will be paid to the effects of drugs on single neurones when the substances are applied by the micro-electrophoretic technique and to evidence relating to the release of chemical substances from nervous tissue when nerves are stimulated.

B) The identification of acetylcholine in brain nerve-terminals

i) Introduction

Many investigators have shown that a substance with pharmacological effects resembling those of acetylcholine may be extracted from mammalian brain (for reviews see Feldberg, 1945, 1957; Hebb, 1957, 1963). However, in most of this early work the ACh was not positively identified either by chemical methods, such as were used by Dale and Dudley (1929) to identify ACh in spleen, or by refined biological assay-methods, such as those used by Chang and Gaddum (1933) to identify ACh in horse intestine, the placenta and in the adrenal glands. A substance resembling ACh is also released from the cerebral cortex (Elliott, Swank and Henderson, 1950; MacIntosh and Oborin, 1953; Mitchell, 1963). However, the cholinomimetic substance released from the cortex may be associated with metabolic processes (Feldberg, 1950; Whittaker, 1963) not directly involved in synaptic transmission. ACh is synthesized by brain extracts and this synthesized product has been chemically identified (Stedman and Stedman, 1937). However, such extracts may also synthesize other cholinomimetic substances (Nachmansohn, Hestrin and Voripaieff, 1949; Middleton and Middleton, 1949; Korey, De Braganza and Nachmansohn, 1951; Henschler, 1956; Morris, 1961). Henschler (1956) demonstrated the presence of a small quantity of ACh (about 60 ng/g) in beef brain extracts, but, since post mortem losses were considerable, no estimate could be given for the amounts of substances originally present. As yet, no conclusive evidence has been presented to show that the cholinomimetic substance originally present in brain is ACh and not a related ester (see also Ambache, 1954;

Pfeiffer, 1959). Such a proof is essential before ACh may be accepted as a transmitter in C.N.S. (criterion 1). The uncertainty regarding the identity of the cholinomimetic substance in brain led Pfeiffer (1959) to speculate that the cholinergic transmitter may be a substance related to acetyl-β-methylcholine, while more recently Hosein and co-workers (Hosein and Proulx, 1960; Hosein and Ara, 1962; Hosein and Proulx, 1962; Hosein, Proulx and Ara, 1962; Hosein and Orzeck, 1963, 1964) have claimed that ACh represents only a small proportion of the cholinomimetic activity of brain extracts, the predominent activity being due to betaine esters and acetylcarnitine.

In the present experiments, advantage has been taken of recently developed techniques for separating brain homogenates into histologically identifiable components (Gray and Whittaker, 1962; De Robertis, Pellegrino de Iraldi, Rodriguez de Lorez Arnaiz and Salganicoff, 1962) under conditions in which synthesis would be minimal. In order to investigate the nature of the cholinomimetic activity of a 'crude mitochondrial' fraction from brain, the technique of parallel biological assay (Chang and Gaddum, 1933) has been applied to both a crude extract and the various active fractions obtained by electrophoretic and chromatographic separation of this crude extract. The 'crude mitochondrial' fraction was used in preference to a whole brain homogenate since the cholinomimetic action of the former is entirely attributable to its content of synaptic terminals (see section IV C). This minimised the possibility that choline esters, which were not associated with synaptic activity, would be extracted and so confound the results.

ii) Theory and Results

Before presenting results obtained on extracts, it is appropriate to consider the usefulness and limitations of the biological and chemical techniques which have been employed to identify ACh. Thus control experiments on cholinomimetic substances and some quantitative theoretical deductions concerning the interpretation of results obtained by parallel assay will be presented in section a).

a) Theoretical aspects of parallel assays and control experiments on cholinomimetic substances

Parallel assays

When the potencies of individual members of a series of choline esters are compared with one another on a variety of biological test-preparations, the estimates are found to differ. Therefore, if a solution containing an unknown choline ester is assayed against known esters on several preparations, the estimated potency will vary, except when the substance used as a standard is identical to the unknown contained in the solution. This is the basis of parallel biological assay, first used by Chang and Gaddum (1933) to identify the choline ester present in horse intestine, in the placenta and in the adrenal gland.

A number of cholinomimetic substances have been reported to be present in brain extracts. These include acetyl-, propionyl- and butyrylcholine (Henschler, 1956, Hosein, Proulx and Ara, 1962), γ-aminobutyrylcholine (Kewitz, 1962), acetylcarnitine (Hosein and Orzeck, 1963, 1964), betaine esters (Hosein and Proulx, 1960; Hosein and Ara, 1962; Hosein, Proulx and Ara, 1962) and imidazoleacetylcholine

(Gruner and Kewitz, 1955). The relative potencies and indices of discrimination (Gaddum, 1959) of some of these substances are shown in Tables 3 and 4 to illustrate that all of those tested were adequately distinguished from one another by parallel biological assay. (The index of discrimination is the potency of the substance relative to ACh on a particular preparation compared with the relative potency on another). In addition it is known that imidazoleacetylcholine can be distinguished from ACh by such assays (Tabachnick, Roth, Mershon, Rubin, Eckhardt and Govier, 1958). Numerous examples of the differentation between other choline esters by means of this technique may be found in the literature (e.g Chang and Gaddum, 1933; Bannister, Whittaker and Wijesundera, 1953; Holmstedt and Whittaker, 1958; Sastry, Pfeiffer and Lasslo, 1960, see also Table 5). To date it would seem that it is possible to distinguish between all choline esters which have so far been examined, including pyruvylcholine (Gaddum, 1963; Paton, 1963) which was earlier reported by Chang and Gaddum (1933) to be indistinguishable from ACh. Thus the method of parallel assay provides a valuable tool in the identification of cholinomimetic substances in brain, particularly when used in conjunction with chromatographic methods, even though the chromatographic Rf values of some of the choline esters may be similar (see Gruner and Kewitz, 1955; Hosein and Orzeck, 1963; Bannister et al., 1953). The value of parallel assays increases with the number of different preparations used. It is obvious that unknown substances possessing a very low potency relative to ACh, unless present in amounts far greater than the ACh in the extract, would not contribute greatly to the observed potency

on different preparations. Thus it is only possible from parallel assays to estimate the proportion of the total activity on a particular preparation which is due to unknown substances. To do this, it must be assumed that the unknown may be quantitatively differentiated from ACh in the parallel assays. This is certainly true for all choline and betaine esters which have so far been examined (see above).

The proportion of the activity due to an unknown substance may be calculated in the following manner from equation (1), given by Gaddum (1959) and adapted to the present problem:

$$A_i = y + x\,Q \qquad (1)$$

where A_i = observed potency of an extract (in terms of ACh) on preparation (i), y is the content of ACh, x is the content of unknown cholinomimetic substance and Q is the relative potency of unknown to ACh on preparation (i).

Let the observed potency of the extract on preparation (i) be 100.

From equation (1):-

$$A_i = 100 = y + x\,Q.$$

Thus $y = 100 - x\,Q$.

Let the unknown be I times more potent, relative to ACh, on preparation (ii) than on preparation (i) i.e. the index of discrimination = I.

The observed potency (A_{ii}) on preparation (ii) is now given by:-

$$A_{ii} = (100 - x\,Q) + x\,Q\,I. \qquad (2)$$

and the ratio of the two observed potencies (R) is given by

the expression

$$R = \frac{100}{(100 - x\,Q) + x\,Q\,I} \qquad (3)$$

Now a consideration of the relative potencies of different choline esters and betaines on a variety of test objects shows that the best differentiation is usually achieved when comparing results obtained on nicotinic receptors, such as those of the rectus abdominis muscle, and muscarinic receptors, such as those of smooth and cardiac muscle. (See Tables 3 and 4). Furthermore the index of discrimination is at least 10, and in many cases is considerably more.

Let it be assumed that the index of discrimination between the unknown cholinomimetic substance and acetylcholine on two preparations is 10, i.e. in formula (2) I = 10. The variation in apparent potency in experiments on brain extracts (see next section and Table 2) was about 20 per cent. Thus the ratio of observed potencies, (R), was 0.8.

Substituting these values for I and R in equation (3):

$$0.8 = \frac{100}{(100 - x\,Q) + 10\,x\,Q}$$

Therefore, $x\,Q = 2.8$.

From equation (1) it is apparent that with A = 100, x Q is the percentage of the potency due to the unknown on preparation (i).

Thus on preparation (i) the maximum percentage of the observed activity (P_i) due to an unknown cholinomimetic is 2.8 per cent.

From equation (2), the maximum percentage of activity (P_{ii}) on preparation (ii) due to an unknown, is given by the expression:

$$P_{ii} = \frac{xQI}{A_{ii}} \cdot 100$$

or $$P_{ii} = \frac{xQI}{(100 - xQ) + xQI} \cdot 100$$

Since I = 10 and xQ = 2.8.

P_{ii} = 22 per cent.

If other values for I are taken, then for I = 5 or 20, it may be calculated that P_i is 6.2 or 1.3 per cent respectively, and P_{ii} is 25 or 21 per cent respectively.

It may be concluded from these calculations that not more than 20-25 per cent of the activity of an extract could be due to an unknown on any preparation, if the extract were compared with ACh on a number of biological test preparations, and the results did not vary by more than about 20 per cent. A proportion, at least, of the variation in actual experiments would be due to experimental error. In biological assays it is not uncommon for this error to be of the order of 10 per cent. The experimental error was not determined in the present experiments, but due to the limited amount of material available for assay, this figure (10 per cent) would not be unreasonable. If allowance for an experimental error of this order is introduced into the preceding calculations, then the maximum percentage of the activity due to an unknown would be halved. This figure (12.5 per cent) may of course still be an over estimate. Nevertheless, it is adequate for the present purposes.

Electrophoresis and chromatography

In addition to detecting the presence of unknown cholinomimetic substances by parallel assays, some of the substances said to be present

in brain could be differentiated from ACh by their electrophoretic or chromatographic behaviour (Table 6). Propionylcholine, n-butyrylcholine, acetyl-β-methylcholine, γ-butyrobetaine methyl ester and acetyl-DL-carnitine had higher Rf values than ACh, whereas γ-aminobutyrylcholine had a lower value. Thus, with the possible exception of acetyl-DL-carnitine all of these compounds would probably have been differentiated chromatographically from ACh. Exept for acetyl-DL-carnitine the electrophoretic mobilities were similar to that of ACh. Thus it was convenient that acetylcarnitine, which was not adequately differentiated from ACh chromatographically with the buffered solvent-system used in the present work, had a much lower electrophoretic mobility, and if present in the extract would have been found in fraction A, whereas ACh was mainly present in fraction B, (see section iii).

In trial experiments with approximately 10 μg of ACh the recovery from paper chromatograms or electrophoretograms, estimated by biological assay of the eluates on the guinea pig ileum, ranged from 80 to 96 per cent. However it was not certain that other cholinomimetic substances would also be recovered in good yield after being subjected to electrophoretic and chromatographic procedures. It was therefore of more relevance to determine the recovery of all cholinomimetic activity, after subjecting the extract to the separation procedures, in addition to examining the crude extract for the presence of substances other than ACh by the method of parallel assay. Results obtained in these experiments are shown in sections b and c.

b) Parallel biological assays on crude extracts

The results obtained in parallel assays performed on two crude

mitochondrial (P2) extracts after incubation with chymotrypsin are shown in Table 7. On all preparations with the exception of toad atria, the apparent cholinomimetic activity, expressed in terms of ACh, did not vary by more than about 20 per cent. However, the estimated activity on toad atria was lower, although this discrepancy was slightly reduced in the presence of physostigmine, which approximately doubled the sensitivity of the preparation to ACh. The low figure is attributed to the presence of an interfering stimulant substance, since low doses of the extract increased the force of contraction of the perfused toad heart (Fig. 3A, E1). In higher doses there was an ACh-like depressant effect followed by a stimulant effect (Fig. 3A, E2). The depressant, but not the stimulant effect, was abolished by treating the extract with alkali. Since the stimulant effect was not seen on the atria (Fig. 3B, E1) it is assumed that the whole heart was more sensitive to the action of the stimulant substance than were the atria. Nevertheless there may have been enough of the stimulant present to counteract partially the action of the ACh in the extract. However, the observation that the cholinomimetic activity of the chromatographically purified fraction was estimated to be the same whether assayed on toad atria or on the other preparations (see Fig. 4 and 5) substantiate the conclusion that the low figure for the crude extract on toad atria was due to an interfering substance.

On the toad rectus untreated with physostigmine, Extract 2 was estimated to contain 20 per cent more acetylcholine than was estimated on the guinea pig ileum. However, if allowance was made for sensitising

substances by comparing the potency of the extract with that of another sample of extract, to which ACh had been added after the alkali labile choline esters had been inactivated by treatment with NaOH (Feldberg and Mann, 1945), then a lower value was obtained (Table 7).

The actions of the extracts were abolished by the administration of atropine (on the ileum, blood pressure and atria) or d-tubocurarine (on the rectus muscle), thus establishing the cholinomimetic nature of the effects.

As was shown earlier, if a cholinomimetic substance other than ACh had contributed more than about 20-25 per cent to the activity, then a variation in the assay figures by more than 20 per cent would be expected. The actual variation was of this order. However, some at least of this variation can be attributed to experimental error (see section a), and there are two additional factors that make it unlikely that any significant proportion of the cholinomimetic activity was due to substances other than ACh. Firstly, the dose-response curves for ACh and the extract were always similar. Secondly, it was noted that the rate of contraction of the toad rectus, which varied greatly with different cholinomimetic substances, was similar for ACh and the brain extract. Examples of substances producing rapid contractures are acetyl-, propionyl-, n-butyryl- and acetyl-β-methylcholine, whereas other substances, such as betaine esters, γ-aminobutyrylcholine, acetyl-DL-carnitine and carbamylcholine produced slower contractures, although the effects at equilibrium were similar. Some examples of fast and slow contractures are illustrated in Fig. 6.

Hosein et al., (1962) claim that most of the cholinomimetic activity of brain extracts was due to substances other than ACh. It was considered possible that the failure to substantiate this claim in the present experiments was due to the method of extraction. Hosein et al.,extracted brain with trichloracetic acid. Therefore in one experiment a 'crude mitochondrial' preparation was extracted in this way instead of by the usual acid and heat treatment. The results of the parallel assays were no different from those obtained by the usual method. Thus there was no evidence that the failure to demonstrate the presence of a cholinomimetic substance other than acetylcholine was attributable to such differences in the method of extraction.

c) Parallel biological assays on the purified fractions obtained by electrophoresis and chromatography

The eluates obtained from the electrophoretograms and subsequently chromatographed contained all the substances which migrated as cations at pH 4, and had an electrophoretic mobility from zero up to four times that of ACh. Thus the eluates might be expected to contain all cholinomimetic substances which have been demonstrated or suspected to occur in brain extracts. The electrophoretic mobilities and chromatographic behaviour of authentic samples of some of these cholinomimetic substances are given in Table 6. In other experiments, anionic substances eluted from electrophoretograms of crude mitochondrial extracts have been examined and contained no additional cholinomimetic activity.

Of the five electrophoretic fractions A, B, C, D and E which

corresponded to substances located - 1 to +3, 3 to 7, 7 to 11, 11 to 15 and 15 to 19 cm respectively from the origin in the direction of the cathode, activity was found in A, B and C. The results on the eluates obtained after chromatography of each of these fractions are plotted in Fig. 4, which shows the total amount of activity (as ACh bromide) in each eluate. In carrying out these tests, sufficient eluate was taken to ensure that the activity of any fraction which contained as little as two per cent of the total activity could be detected.

Fraction A (-1 to +3 cm, Fig. 4 A)

Fraction A contained unionised substances and substances having a low anionic or cationic mobility at pH 4. After chromatography of this fraction, eluates of the strips corresponding to Rf 0.1 - 0.2 and 0.2 - 0.3 caused the guinea pig ileum to contract. The total activity of these strips was equivalent to 0.5 and 1.0 μg of ACh bromide respectively. They also caused prolonged contractures of the toad rectus muscle and depressed the size of the contractions of toad atria. The effect on toad atria was not reproducible with repeated doses. All these effects were not cholinomimetic in nature since they were not blocked by atropine on the guinea pig ileum or by d-tubocurarine on the rectus muscle. The effects of these eluates were attributed to the presence of triethylamine, for two reasons. First, the active eluates had an odour of triethylamine and the presence of this compound was substantiated by chromatography in a pyridine/acetic acid/water/1-butanol (3:8:11:33, by vol.) solvent system: a strong ninhydrin-positive spot appeared at the same Rf as that for triethylamine chloride (0.7). Second, the biological effects of triethylamine were identical with those of the eluates. In

another experiment in which the brain extract was subjected to electrophoresis and chromatography in buffer systems containing pyridine in place of triethylamine (see below), similar bands of biological activity were obtained. In this instance, the activity was attributed to pyridine, which had biological activity similar to that of the eluates, and was detected spectrophotometrically.

Fraction B (+3 to +7 cm, Fig. $4B_1$, B_2)

This was the most interesting of the fractions obtained on electrophoresis, since it should have contained most of the choline esters and betaine esters present in the extract (Table 6).

The results obtained on fraction B are illustrated in Fig. $4B_1$ and $4B_2$. Three peaks of activity were noted. The Rf value of the largest of these peaks (Peak 2) was about 0.5 which corresponded to that of ACh (Table 6). Fig. $4B_2$ illustrates how the activity was sharply localised to one band at Rf 0.49. In Fig. $4B_1$ the activities of this band and those of the two adjacent bands were pooled to give the single value (Peak 2) which is plotted at Rf 0.49. The estimated potency of this band (12.4 to 14.2 µg) did not vary by more than 15 per cent on any of the four preparations (guinea pig ileum, toad rectus, toad atria and rat fundus) on which it was tested. From the equations given earlier it can be calculated that, assuming a discrimination index of 10, the maximum proportion of the activity which could have been due to substances other than ACh and which exhibited identical chromatographic behaviour, was approximately 17 per cent. It is of particular interest to note that toad atria gave a much closer potency estimate to that obtained on the other preparations, than was found when testing crude extracts.

The actions were completely abolished by appropriate pretreatment of the preparations with atropine, hyoscine or d-tubocurarine. Furthermore, comparisons of the dose-response curves on each of the preparations where sufficient data were obtained revealed no differences from those of authentic acetylcholine (Fig. 5), and on the rectus abdominis preparation the time courses of the contracture and recovery were similar to those of acetylcholine(Fig. 7). Fig. 5a and 5b show that physostigmine potentiated the actions of authentic ACh and the brain extract on the toad rectus to the same degree. This may be taken to indicate that both ACh and the cholinomimetic substance in brain are broken down by cholinesterase to a quantitatively similar extent. This battery of observations thus provides substantial evidence that the activity of the band was due to ACh and not to a related ester.

However, interpretation of the chromatographic examination of fraction B was complicated by the additional peaks of activity (Fig. $4B_1$, peaks 1 and 3) at Rf 0.31 and 0.96, representing the equivalent of about 3 and 1 μg of ACh bromide respectively on each of the four preparations. Since there was little variation in the estimates from preparation to preparation and because the effects were blocked by atropine, hyoscine or d-tubocurarine, it seemed probable that the activity of these fractions was also due to ACh. The different locations of the ACh on the chromatograms were attributed to binding by the other components of the mixture. When the residuum of these two active samples was rechromatographed in the original solvent system, and the eluates assayed on the toad atria, all of the activity was recovered from a band corresponding to Rf 0.5 (Fig. 8). The apparent low recovery (compare Fig. 8 with Fig. 4) is due to the removal of some of the sample for the

assays described above. Thus the activity of all three peaks in fraction B may be attributed to ACh.

It was estimated that 21.4 μg (as ACh bromide) of active material were applied to the papers for electrophoresis. This figure makes allowance for the amount of material lost in cutting off the side strips and is the average of the values determined on the guinea pig ileum, the toad rectus muscle and guinea pig atria. The total activity attributable to ACh, which was recovered from fraction B after chromatography, was estimated to be 16.4 μg on the guinea pig ileum, 18.5 μg on the rat fundus, 17 μg on the toad rectus and 18.3 μg on toad atria. The average of these values is 17.6 μg which represents 78 per cent of the applied material. It is particularly significant that there was no activity at the Rf corresponding to propionyl- or n-butyrylcholine (Table 6).

The Rf of γ-aminobutyrylcholine is about 0.2 (Table 6) and no activity in fraction B was detected in this region. γ-Aminobutyrylcholine was quite inactive on the guinea pig ileum and toad atria (Table 3), but the toad rectus was sensitive to about 25 to 50 μg per ml. Since only one tenth of the sample was tested it may be concluded that the sample contained less than 250 to 500 μg of this relatively inactive substance. Since the amount of the extract analysed was equivalent to 16.5 g of fresh brain, it may therefore be concluded that the crude mitochondrial fraction from brain contained less than 15 to 30 μg of γ-aminobutyrylcholine per gram of brain. Kewitz (1962) estimated the γ-aminobutyrylcholine content of rat brain to be 142 x 10^{-9} moles/brain. This is equivalent to 21 μg of γ-aminobutyrylcholine chloride per gram of brain, assuming a brain weight of 1.5 g, which is the approximate weight of the fore brain of rats weighing about 250 g. Thus the rectus was

barely sensitive enough to detect γ-aminobutyrylcholine at concentrations of this order.

Results similar to those described in this section were also obtained in experiments in which pyridine replaced triethylamine in the solvent systems (see below).

Fraction C (+7 to +11 cm, Fig. 4C)

Fraction C contains substances of slightly greater ionic mobility than fraction B. The only activity detected in the chromatographic subfractions of fraction C was cholinomimetic in nature, since it was abolished by the usual blocking agents. Furthermore, it appeared to be ACh since on three preparations there was no significant difference in the activity when compared with authentic ACh, and the Rf of the active band coincided with that of authentic ACh (Fig. 4C). The activity of this band was equivalent to 3 μg of ACh when assayed on the guinea pig ileum. It was estimated to be 2.6 μg on toad atria and 2.2 μg on the toad rectus. The average of these values is 2.6 μg and represents a further 12 per cent of the active material taken.

Thus the recovery of ACh in fractions B and C represents 90 per cent of the amount of cholinomimetic substance taken from the crude extract, and is similar to the recovery of authentic acetylcholine (see section ii,a).

Fractions D and E (+11 to +19 cm)

Both of these fractions were completely inactive on all tests.

d) Experiments in which pyridine-containing buffer solutions were employed

When electrophoresis and chromatography was carried out in buffered solutions containing pyridine, instead of triethylamine as in the previous experiments, the results were more complicated. However, the main finding was reproduced: all of the cholinomimetic activity, i.e. effects on the guinea pig ileum, toad rectus and toad atria which were blocked by atropine or d-tubocurarine, was recovered from a single band, corresponding in electrophoretic mobility, chromatographic behaviour and parallel assay (Fig. 9) to ACh.

The Rf of ACh in this solvent system was 0.5 - 0.6. In the chromatograms of all cationic fractions and the neutral fraction there was an additional peak of activity on the toad rectus (see Fig. 9). These additional active regions of the paper chromatograms had higher Rf values (0.65 - 0.8) than ACh and were absent from the chromatograms of anionic fractions. The activity was not blocked by d-tubocurarine, which shows that it was not caused by a cholinomimetic substance. When sheets of S.S. paper were eluted with pyridine-containing buffer, and the eluates chromatographed as before, a similar band of activity was obtained (Fig. 10) and the active material behaved as a cation on electrophoresis at pH 4. Figure 11 illustrates the effects of an eluate from chromatographic paper on the rat fundus, toad rectus and toad atria. The activity was increased when the paper was first subjected to electrophoresis. Thus it is concluded that this biologically active material did not originate from the brain extract. The residue obtained by freeze-drying a sample of buffer which had been refluxed on a boiling

water bath for 0.5 to 1 hr had a similar effect on the rectus, but a sample which had not been refluxed had no activity. The amount of activity obtained after refluxing varied with the commercial brand of acetic acid from which the buffer was constituted, but pure acetic acid or pyridine possessed no activity when they were refluxed alone. Active material was also obtained when glass paper (Whatman, Type GF/B) or silica gel was eluted. These facts suggest that the active compound is formed by the interaction of pyridine with anionic substances from paper, glass paper, glass, silica or from the reagents themselves under suitable conditions. It was noted that when the active residues were injected into the Ringer solution in the organ bath there was marked foaming. Since detergents, such as cethyl-trimethylammonium ('Cetavlon') in low concentrations also produced prolonged contractures of the rectus, (Fig. 12), it is possible that the unknown compound was of a similar type. However, no attempt was made to identify pyridine in the eluates of all of the active fractions from chromatograms of brain extracts.

It will be recalled (see page 35) that when brain extracts were electrophoresed and chromatographed in the triethylamine-containing buffer, the activity at Rf 0.1 - 0.3 of the neutral fraction (A) was attributed to the presence of triethylamine (Fig. 4A). Activity at a similar Rf in the neutral fraction (Fig. 9A) was found when the separations were carried out in the pyridine-containing buffer. In this case, the action of the eluate could be attributed to pyridine. The pyridine was detected and assayed spectrophotometrically and Fig. 13 illustrates the ultraviolet absorption spectrum of the active eluate in comparison with that of pyridine. The pyridine was probably associated with ethano-

lamine phosphate, which was shown to be present by chromatographic methods.

Biologically active material could also be eluted from unwashed chromatographic paper by solvents which did not contain an organic base. This material could be removed by careful and prolonged washing of the paper (section III B), but its nature has not been determined.

iii) Discussion

Ambache (1954) obtained inconclusive resuts when attempting to resolve the question of the identity of the cholinomimetic substance in guinea pig and rabbit brain by comparing the activity of the extracts on the frog rectus and guinea pig ileum. Welsh (1943) observed that the ACh equivalent of rat brain was consistently less when estimated on frog heart than on the heart of Venus mercenaria. n-Butyrylcholine is said to be present in brain extracts (Holtz and Schumann, 1954; Hosein, Proulx and Ara, 1962; Hosein and Orzeck, 1964), but these results are not supported by the observations made by Henschler (1956) or by Keyl (1963). Recently, Kewitz (1962) has produced evidence for the presence of considerable quantities of γ-aminobutyrylcholine and an unidentified choline ester in brain extracts. Hosein and co-workers (Hosein and Proulx, 1960; Hosein and Ara, 1962; Hosein and Proulx, 1962; Hosein, Proulx and Ara, 1962; Hosein and Orzeck, 1963, 1964) consider that acetylcholine contributes only slightly to the cholinomimetic activity of brain extracts, and claim that betaine esters related to γ-butyrobetaine and acetylcarnitine were largely responsible for the activity.

The present experiments were designed to shed some light on the identity of the cholinomimetic substance present in synaptic terminals

in brain. The cholinomimetic activity of the 'crude mitochondrial' preparation from brain is associated with the presence of nerve terminals, and represents about 70 per cent of the total activity of a homogenate (Whittaker, 1959; Gray and Whittaker, 1962; De Robertis, et al., 1962; section IV C). This crude mitochondrial preparation was used to increase the likelihood that any cholinomimetic substance detected would be derived from the nerve terminals and that presumably it would be associated with synaptic activity rather than with metabolic functions of the tissue (Feldberg, 1950).

The results indicated that the cholinomimetic activity of the terminals could be attributed to ACh and not to a related substance. This conclusion is based upon a number of criteria. Parallel assays of the crude extract upon several isolated organs showed that, except on toad atria, the activity, estimated relative to ACh, did not vary by more than about 20 per cent. The low value obtained on toad atria was attributed to an interfering stimulant substance which could be demonstrated on the isolated, perfused toad heart. Furthermore, after electrophoretic and chromatographic separation of the extracts, assays on toad atria yielded results comparable with those obtained on other preparations. The cholinomimetic nature of the activity was demonstrated by the blocking actions of atropine, hyoscine or d-tubocurarine. Inactivation of cholinesterase by treatment with eserine or neostigmine did not influence the result, which indicated that the active substance and ACh were inactivated by cholinesterase to a quantitatively similar degree. Qualitatively, as well as quantitatively, the effects produced by the extract and ACh were similar. This was particularly significant on the toad rectus, where the actions of many substances, including choline esters, betaine

esters and acetylcarnitine, differed from that of ACh in the rate at which the contractures reached equilibrium. The slopes of the dose-response curves obtained for the extracts were similar to those of ACh in all experiments in which sufficient data were obtained.

These results on the crude extracts alone provide substantial evidence that the cholinomimetic activity was due to ACh and not to a related substance. The validity of this conclusion is based upon the assumption that all cholinomimetic substances can be differentiated from one another by the method of parallel assay. This assumption has been validated for all cholinomimetic substances so far investigated (Chang and Gaddum, 1933; Bannister, Whittaker and Wijesundera, 1953; Holmstedt and Whittaker, 1958; Sastry, Pfeiffer and Lasslo, 1960; Gaddum, 1963; Paton, 1963). In addition, it has been shown that γ-butyrobetaine methyl ester, acetyl-DL-carnitine, γ-aminobutyrylcholine and γ-propiobetaine methyl ester, can also be differentiated by parallel assays. It was shown that for substances with an index of discrimination of ten, if about 20 per cent of the activity were due to compounds other than ACh, they would have been detected in the present experiments. Although Hosein and Orzeck (1963) state that the potency of acetylcarnitine on the frog rectus was only slightly less than ACh, in the present experiments it was found to have an extremely low potency on all preparations. However, the present observations are in agreement with those of a number of other investigators (for references, see Fraenkel and Friedman, 1957). Some of the observations made on the acetylcholine-like effects of acetylcarnitine are confounded by the presence of acetylcholine as a contaminant in the preparations (Fraenkel

and Friedman, 1957).

However, the conclusions in the present investigations are not based merely upon parallel assays on the crude extract, but also upon the electrophoretic and chromatographic behaviour of the active substance. Chromatography was carried out in buffered solvent-systems in an attempt to reduce the incidence of aberrant mobilities which are known to occur in unbuffered systems (see Whittaker, 1963). When the extract was subjected to electrophoresis at pH 4, most of the original biological activity was recovered from a band corresponding to ACh in ionic mobility (Fraction B). A small amount was recovered from a slightly more cationic band (Fraction C), but this was presumably due to the failure of all electrophoretograms to run in an absolutely identical fashion. There was some activity in the band situated between -1 to +3 cm from the origin (Fraction A). However, the activity of this band was not cholinomimetic in nature since it was not affected by treatment of the biological preparations with atropine or d-tubocurarine. Triethylamine was shown to be present in the active eluates and the activity of these eluates was attributed to triethylamine. Presumably triethylamine from the buffer had formed salt linkages with anionic groups of some of the components of the extracts.

Chromatography of the eluates obtained from the electrophoretograms showed that 80 per cent of the active cholinomimetic substance had an Rf value which corresponded to that of ACh (Rf 0.5) in the solvent system. The active substance eluted from this region of the chromatogram behaved in a qualitative and quantitative fashion like ACh in all tests. This substance was also applied electrophoretically

to single Renshaw neurones in the cat spinal cord (see section IV D) and its action was indistinguishable from that of authentic ACh. Thus, not only did the substance correspond to ACh chromatographically and in parallel assays on isolated organs, but is also corresponded to ACh in tests on neurones in the central nervous system.

Fifteen per cent of the recovered cholinomimetic activity was located on the chromatograms at Rf 0.3 and a further 5 per cent at Rf 0.96. The parallel assays suggested that the activity of these fractions was also due to ACh. This conclusion was substantiated by the evidence that on rechromatography of both fractions, the active substance was eluted from the band having the same Rf as ACh (Rf 0.5). Since the electrophoretic separation was carried out in an acetate buffer all cations should have been present as the acetates. Thus the complex behaviour of the ACh on chromatography could not have been due to mixtures of anions (Whittaker, 1963), but some of the components of the extract, although cationic at pH 4, may have contained anionic groups capable of binding some of the ACh in preference to triethylamine in the chromatographic system.

Ninety per cent of the original cholinomimetic activity of the crude extract was recovered after purification by electrophoresis and chromatography, and all of this could be attributed to ACh. A similar loss occurred when ACh was subjected to the same procedures. Therefore, it seems unlikely that the failure to detect substances other than ACh was due to losses during separation. Furthermore, the amounts of eluates tested would have enabled the detection of as little as about

2 per cent of the activity at an Rf different from that of ACh.

It should be emphasised that the techniques used in the present investigation would not have detected the presence of even relatively large amounts of very inactive substances such as γ-aminobutyrylcholine, acetylcarnitine or choline. However, the results clearly demonstrated that the cholinomimetic activity estimated on the guinea pig ileum and atria, rabbit atria, rat blood pressure, toad rectus and atria and rat fundus was not due to these substances to any marked degree.

Thus it is concluded that the cholinomimetic action of extracts of synaptic terminals from rat brain is due to acetylcholine and the conclusions of Hosein and co-workers (loc.cit.) that the cholinomimetic activity of brain extracts is due to other substances is not substantiated. Since this work was completed, several groups (Pepeu, Schmidt and Giarman, 1963; McLennan, Curry and Walker, 1963; Grossland and Redfearn, 1963) have produced convincing evidence that the results obtained by Hosein et al., may have been due to the aberrant behaviour of ACh in the unbuffered solvent-systems which were used.

A serious objection to these present conclusions would arise only if it could be shown that naturally occurring substances, for instance the betaine coenzyme A esters described by Hosein and co-workers (Hosein, Proulx and Ara, 1962; Hosein and Orzeck, 1964), could not be differentiated from ACh in all the tests which have been applied in this investigation. This is considered to be unlikely for the reasons given above. Furthermore, the betaine esters are not hydrolysed by cholinesterase (Hosein, Proulx and Ara, 1962), whereas in the present

work it was shown that inhibition of cholinesterase did not affect the apparent acetylcholine content of the extracts, which implies that the extracts and acetylcholine were hydrolysed by cholinesterase to the same extent.

Hosein and Orzeck (1964) found that there was more n-butyrylcholine than ACh in extracts from calf brains. In the present experiments less than 2 per cent of the cholinomimetic activity in rat brain terminals was due to n-butyrylcholine: since n-butyrylcholine is about three times as potent as ACh on the toad rectus (Table 3), it may be concluded that the ratio of n-butyrylcholine to ACh was less than 1:100 by weight. The high proportion of n-butyrylcholine found by Hosein and Orzeck (1964) in calf brain may be due to a rapid and marked post mortem loss of ACh, coupled with the post mortem synthesis of n-butyrylcholine (Henschler, 1956).

The experiments described in this section have demonstrated that ACh is a naturally occurring substance in the central nervous system. It therefore satisfies the first requirement of a synaptic transmitter (criterion 1, see page 20). In the next section (IV C) other evidence relating to criteria 1, 2 and 3 (see page 20) will be presented, and in later sections some experimental support for the remaining criteria will be discussed.

C) Evidence concerning transmitter function derived from studies of subcellular distribution

i) Introduction

Criterion 1 (section IV A, see page 20) states that a substance considered to be a transmitter should be stored at those sites from which it is supposed to be liberated. Synapses in the central nervous system are anatomically well defined structures, and a characteristic feature is the occurrence of numerous 'synaptic vesicles' (De Robertis and Bennett, 1954; De Robertis, 1958) within the presynaptic terminals (De Robertis, 1959; Palade and Palay, 1954; Palay, 1956, 1958; Whittaker and Gray, 1962). It has been said that the vesicles contain the stored transmitters (see previous references and Eccles, 1964), but MacIntosh (1963) has questioned this interpretation and supports the suggestion that the observed structures may not in fact be vesicular in nature bu a complex system of tubules (see also, Robertson, 1960, 1961) and that these structures may not store the transmitters. Nevertheless, it could be expected that the transmitters would be concentrated in the terminals to a greater extent than in the remainder of the neurone, and, accepting for the moment the usual interpretation of the function of synaptic vesicles, the transmitters might be even more concentrated in the vesicles. A study of the localization in different portions of the neurone of substances of interest as possible transmitters could yield valuable information in relation to criterion 1 (see page 20) regarding the function of these substances.

The first step in this direction was taken by Hebb and Whittaker (1958) and Whittaker (1959). They applied the techniques of differential

and density-gradient centrifugation to an analysis of the subcellular distribution of ACh and cholinesterase in brain homogenates. They found that most of the bound acetylcholine-like substance, which has now been shown to be ACh (section IV A), and choline acetylase was located in a subcellular fraction lighter than mitochondria. It was originally suggested that this fraction contained mainly vesicles of a size comparable with that of synaptic vesicles (Whittaker, 1959), but later it was shown that the fraction contained mainly pinched-off nerve-endings (Gray and Whittaker, 1962) and that the vesicles were produced as a consequence of the fixation procedure. These findings were confirmed by De Robertis, Pellegrino de Iraldi, Rodriguez de Lorez Arnaiz and Salganicoff (1962), who used a slightly different technique. De Robertis et al., distinguished two nerve-ending-fractions, one of which contained the ACh and acetylcholine esterase (the cholinergic nerve-ending-fraction) and another which contained little ACh (the non-cholinergic nerve-ending-fraction). Very recent evidence has indicated that the ACh is further localized in the synaptic vesicles (De Robertis, Salganicoff, Zieher and Rodriguez de Lorez Arnaiz, 1963; De Robertis, Rodriguez de Lorez Arnaiz, Salganicoff, Pellegrino de Iraldi and Zieher, 1963; Whittaker, Michaelson and Kirkland, 1963, 1964). There is, however, a dispute between these two groups of workers regarding the distribution of the enzyme concerned with the synthesis of ACh, and at present it is not possible to decide between the two opposing views.

Thus the localization of ACh fulfills criterion 1. It was therefore of interest to determine the subcellular distribution of other

substances which are candidates for the role of transmitter, and the results of such a study are reported in the following part of the thesis.

ii) Results

The various subcellular fractions obtained in the present investigation (see section III A) were similar in gross appearance to those obtained in previous studies in which fractionation was monitored electronmicroscopically (De Robertis et al., 1962; Whittaker, 1959; Gray and Whittaker, 1962). Since the amounts and distribution of ACh were also similar (see below) it seems justifiable to conclude that the subcellular particles were also comparable. Before discussing the subcellular distribution of the various substances, it will be convenient to describe first the characteristics of the particles which are obtained, and to discuss certain theoretical aspects of the distribution.

a) Types of subcellular particle

The method of obtaining the subcellular particles is described in section III A (see also Tables 1 and 2). The primary fractions (P1, P2, P3 and S), obtained by differential centrifugation, are apparently similar morphologically, regardless of the minor differences in technique employed in the two basic methods (see Gray and Whittaker, 1962 and De Robertis et al., 1962).

P1, the 'nuclear' fraction, contains cellular debris, nuclei and any erythrocytes which were present in the original homogenate. It is the heaviest of the subcellular fractions, and is sedimented in low centrifugal fields.

P2, the 'crude mitochondrial' fraction contains a wide variety of particle types. Among these are included mitochondria, nerve-ending-

particles, myelin fragments and a number of microsomes.

P3, the 'microsomal' fraction has not yet been analysed in detail by electronmicroscopy. However, it seems to contain predominently vesicles of microsomal dimensions.

S, the supernatant or 'cytoplasmic' fraction, contains the soluble cytoplasmic components and ribosomes which have not been previously sedimented.

Fractionation of P2 in density-gradients yields the following subfractions: the subfractions obtained by method (a)(Table 1) have no suffix but the subfractions obtained by method (b)(Table 2) are denoted by the suffix 1 (see also Fig. 1).

A or A_1 contains mainly myelin fragments.

B is a complex formed by small fragments of nerve-endings, curved membranes of synaptic type, some myelin fragments and vesicles of microsomal size.

C consists mainly of nerve-endings containing synaptic vesicles.

D also contains numerous nerve-endings.

B_1 contains all those elements present in B, C and D.

E or C_1 consists almost entirely of free mitochondria.

This description is based upon the electronmicroscopic pictures of De Robertis et al., (1962) and Gray and Whittaker (1962).

b) Theoretical aspects of the distribution

There are two ways in which a substance may exist in a tissue; it may be 'free' or it may be 'bound'. In the present context the term 'bound' will be taken to indicate the state in which a substance, when present in a tissue, requires some form of treatment, other than simple

homogenization, to liberate it into the supending medium. Thus a bound substance may be sequestered within subcellular particles from which it cannot diffuse, it may be physically adsorbed onto cellular or subcellular membranes, or it may be in a state of chemical combination with components of membranes or other subcellular structures. By exclusion, the term 'free' will be applied to soluble cytoplasmic material, evenly distributed through the cytoplasm which is not so bound and is free to diffuse from the tissue into the surrounding medium under the conditions of the experiments. In vivo, such 'free' substances may not be able to diffuse from cells due to the operation of ionic pumps.

When a tissue is homogenized and the homogenate centrifuged a free substance will distribute between a sediment and the supernatant fluid in proportion to their relative fluid contents. As a first approximation, the fluid content may be assumed to be related to the relative volumes of the sediment and the supernatant fluid. This approximation will tend to overestimate the amount of free substance in the sediment, since the particles themselves must occupy a significant proportion of the total volume. Since the volumes of the various subcellular fractions obtained from brain homogenates varied considerably (for instance, the microsomal fraction consisted of a small, dense pellet, whereas the crude mitochondrial fraction occupied a relatively large volume and contained a large amount of fluid), a misleading interpretation of an unequal distribution of substances between the various fractions could easily arise, unless some account is taken of their relative volumes.

An approximate estimate of the distribution to be expected of a freely diffusible, cytoplasmic substance was obtained by keeping a record of the volumes of the sediments and their supernatant fluids

at each stage of the fractionation.

As an example of the type of simple calculation involved, in one experiment the volume of the unwashed P1 sediment was 14 ml and the volume of the supernatant fluid was 38 ml. Thus the maximum amount of diffusible substance remaining in the sediment would be 27 per cent. After resuspension of P1 and centrifuging, the volume of the sediment was 4 ml and that of the supernatant fluid was 22 ml. Therefore the maximum amount of diffusible substance remaining in P1 would be 4 per cent, and the amount in the pooled supernatant fluids would be 96 per cent. Similar calculations were applied at each stage of the fractionation.

The results of four experiments (Method b) were pooled to give the distribution for a freely diffusible substance which is illustrated in Fig. 14. The ordinate plots the percentage recovery, expressed as the amount of material in a particular fraction as a percentage of the total amount in P1 + P2 + P3 + S. The subcellular fractions are indicated along the abscissae. The most striking thing about this distribution is the large amount (80 per cent) of freely diffusible material in the cytoplasmic fraction (S). This, of course, is not an unexpected result, since the volume of S was obviously much greater than that of the other fractions. A more interesting finding, and one that is relevant to the distribution of potassium and amino acids, is the observation that there is a peak recovery, among the particulate fractions, in the crude mitochondrial fraction (P2). The recovery in P2 represents 14 per cent of the total recovery. In contrast with this result, the recovery in the remaining fractions (P1 and P3) and subfractions (A_1, B_1, and C_1) is very low (5, 1.3, 0.75, 0.65 and 0.05 per cent respectively).

Now, fractions P2 and B_1 contain the pinched-off nerve-endings, complete with their entrapped cytoplasm. Any soluble cytoplasmic material contained within them may not be able to diffuse freely into the surrounding medium due to the restrictive effect of the enclosing cellular membranes. This fact introduces a complication into the theoretical distribution of soluble cytoplasmic material, originally evenly distributed throughout the cell, and the amount of such material in P2 and B_1 will tend to be underestimated to an extent depending upon the relative volumes of cytoplasm in the nerve-endings and the remainder of the cell.

The subcellular distribution of potassium may provide an indication of the volume of cytoplasm trapped within the nerve endings, although the rapid entry of potassium into the surrounding medium (Elliott and Bilodeau, 1962) may cause this volume to be underestimated. Fig. 14 also illustrates the results which were obtained for potassium. Twenty four per cent of the total potassium was recovered from P2. This value is somewhat greater than the theoretical value for a freely diffusible cytoplasmic substance, and the difference between the two values is attributed to sequestering of the potassium within subcellular particles. This conclusion is substantiated by the observation that, on subfractionation of P2, 31 per cent of the P2-potassium was recovered from A_1, B_1 and C_1, whereas the recovery to be expected of a freely diffusible substance was calculated to be only 11 per cent. If the remaining 69 per cent of the P2-potassium is assumed to represent that contained in the extra-particulate fluid, the amount in the extra-particulate fluid

may be calculated to be 24 x $\frac{69}{100}$ = 16.5 per cent of the recovered potassium. This value is just greater than the 14 per cent calculated theoretically for a freely diffusible substance (see Fig. 14). Since 14 per cent was the maximum value for a freely diffusible substance, it is concluded that a certain amount of the potassium had leaked from the particles on subfractionation, a not unreasonable explanation.

Katzman and Leiderman (1953) have shown that about 25 per cent of the total potassium in brain is unable to exchange with radioactive potassium, either in vivo or in vitro. However, it is not clear to what extent this non-exchangeable potassium contributed to the amounts recovered from the crude mitochondrial fraction since it seems unlikely that it is extracted under mild acid conditions, such as were used in the present experiments (Katzman and Leiderman, 1953).

Thus the distribution of potassium provides an indication of the distribution to be expected of a substance which is evenly distributed throughout the cellular cytoplasm and is not confined to any special subcellular particle.

An alternative method by which substances could be bound in cells is by non-specific binding to proteins, which are not especially characteristic of any particular subcellular component. An assymetric distribution of protein among the various subcellular fractions would lead to an assymetric distribution of bound material, and so could lead to an incorrect interpretation. Some control over this possibility may be obtained by comparing the distribution of a substance under study, with that of protein or of nitrogen. The results of such comparisons are expressed as the relative specific activities (RSA), which may be defined as the ratio of the percentage recovery of substance in a parti-

cular subcellular fraction to the percentage recovery of nitrogen in the same fraction.

c) Acetylcholine

The distribution of acetylcholine in brain homogenates was similar regardless of the species or the method of fractionation. Some of the results are detailed in Table 8 and Fig. 15. More thorough homogenisation, in which the clearance between rod and annulus was reduced to 17 μ, did not affect the overall distribution. Method a) (see Table 1) tended to give slightly higher values for the microsomal fraction (P3) than did Method b) (see Table 2), but the ACh content of P2 always greatly exceeded that of P3. In the absence of physostigmine in the sucrose media there was very little ACh in the cytoplasmic fraction. In the presence of physostigmine the amount of ACh recovered from the cytoplasmic fraction (S) was about 30 per cent of the total (seven experiments on rats and guinea pigs) but otherwise the distribution was not altered. The apparent difference between the recovery of ACh in P1, P2 and P3 in the presence of physostigmine (column 1, Table 8) and the recovery in the absence of physostigmine (column 2, Table 8), was not significant ($P > 0.5$) when taken in conjunction with other results in which P2 was not subfractionated.

In three experiments, anaesthesia in guinea pigs raised the level of ACh in P2 to a similar amount to that found in anaesthetised rats (column 1,2, Table 8). Similar effects have been noted by others (Crossland and Merrick, 1954). The brain from a sheep killed by exsanguination had a very small amount of ACh in P2 and in another experiment on a sheep anaesthetised with pentobarbitone the amount was still very

small (0.26 μg/g compared with 0.07 μg/g in the unanaesthetised sheep).

As first shown by Whittaker (1959), the crude mitochondrial fraction (P2) contained most of the particle-bound ACh in brain. On subfractionation of P2 in the density gradient most of the ACh was recovered from fraction B + C when Method a) was used, or from fraction B_1 when Method b) was used (Table 8). The variation in the relative amounts of ACh in fraction B and C (see Section III A) is illustrated by the following figures. In four experiments the ACh content of B was 0.52, 0.30, 0.18 and 0.24 μg/g respectively and the corresponding values for C were 0.12, 0.14, 0.27 and 0.23 μg/g. The total ACh in these two fractions varied very little from a mean value of 0.5 μg/g (0.64, 0.44, 0.45, and 0.47 μg/g, respectively).

The specific localisation of ACh in these fractions is also shown by examination of the relative specific activities (Table 8). The highest RSAs were found in B + C or B_1. It is of interest to note that in fractions B and C the relative amounts of nitrogen varied in a manner similar to that of ACh. The RSA for fraction C was greater than that for B. The RSA for P2 was invariably greater than that for P3. This last point is extremely important when the results are compared with those obtained for 5-HT.

When the distribution of ACh (Table 8 and Fig. 15) is compared with that of potassium (Fig. 14) it is obvious that the distribution of ACh does not correspond to that of an evenly distributed cytoplasmic constituent. Thus the conclusion that ACh is especially concentrated in nerve-terminals seems adequately justified.

d) Substance P

In some of the early experiments it was apparent that the ACh-

like activity and 5-HT-like activity of P2 extracts which were not first incubated with chymotrypsin were only partially blocked by atropine or BOL-148 respectively (Ryall, 1961, unpublished results). These observations led to a study of the subcellular distribution of substance P-like activity, which it was thought may have produced the spurious results. It was considered that, although the amount of substance P present in a homogenate was not likely to affect seriously the assay for ACh or 5-HT (see also Whittaker, 1962), a different distribution of the various substances between the subcellular fractions could give rise to misleading results.

The results on substance P are presented in Table 9. Most of the substance P was recovered from the crude mitochondrial fraction (P2) and when this was subfractionated, the highest proportion of the activity was in the nerve-ending fractions B_1, or B + C. There was very little difference in the results in different species, with different intensities of homogenisation or with different methods of fractionation, except that Method a) yielded rather more substance P in P3 than did Method b). However, a similar result was obtained for ACh (Table 8). The highest RSA on subfractionation of P2 occurred in the B_1, or B + C fractions. The microsomal fraction (P3) had a slightly higher RSA than P2, and in this respect the distribution of substance P differed slightly from that of ACh. Thus in general, the distribution of substance P is very similar to that of ACh and it is significant that fraction D, the non-cholinergic nerve-ending-fraction of De Robertis et al., (1962) contained very little substance P.

e) 5-Hydroxytryptamine (5-HT)

In contrast with results obtained by Michaelson and Whittaker

(1963), it was found that the subcellular distribution of 5-HT differed in certain important respects from that of ACh. Most of the experiments were carried out in rats using Method a) and Table 10, column 1 shows the average results obtained in four experiments. In these experiments 34 - 70 per cent (average 46 per cent) of the 5-HT was in the cytoplasmic fraction (S). However, the most interesting finding was that there was almost as much 5-HT in P3 as in P2. Furthermore, the RSA for the latter was about one-third of that for P3. This is in marked contrast with the results obtained for ACh (Table 8, column 1) where both the percentage recovery and the RSA were greater for P2 than for P3. It was of considerable importance to establish the statistical significance of this difference in the distribution of 5-HT and ACh. For 5-HT (RSA for P3)/(RSA for P2) in the four experiments shown in Table 10, column 1, was 2.37 ($\pm$ standard error 0.23). The ratio for acetylcholine in the same experiments was 0.66 $\pm$ 0.09. The difference between these two mean values was highly significant ($P < 0.001$). Similar results were also obtained in guinea pigs (Table 10, column 2, 3 and 4), whether Method a) or b) was used in the preparation of the subcellular fractions.

In one of the experiments on guinea pigs, iproniazid (1 mM) was added to the sucrose media throughout the separation. The distribution of 5-HT in the primary fractions P1, P2, P3 and S resembled that found in the absence of iproniazid, although the amount of 5-HT recovered was higher, but differences were noted on subfractionation of P2. In the absence of iproniazid (Table 10, columns 1, 2 and 3) there was relatively little 5-HT in fractions more dense than C (Method a)) or B_1 (Method b)). However, in the presence of iproniazid most of the 5-HT from P2 was recovered from the pellet at the bottom of the density-gradient.

This is fraction C_1 which normally contains free mitochondria (Gray and Whittaker, 1962) and it had the highest RSA recorded in any experiment. Despite this difference between the distribution of 5-HT in experiments in which iproniazid was used and those in which it was not, there was no difference noted in the distribution of ACh or substance P. On attempting to repeat this experiment on two subsequent occasions it was noted that there was an obvious aggregation of the particles, and the appearance of thelayers in the density-gradient differed markedly from those obtained in the absence of iproniazid. Therefore further experiments along these lines were discontinued.

Michaelson and Whittaker (1963) recently reported that 5-HT is absent from fraction P3. It was therefore of interest to attempt to explain the discrepancies between these results. Since these authors used a fluorimetric method of assay for 5-HT, it was thought worthwhile to compare assays for 5-HT by fluorimetric and biological methods. In a preliminary experiment fluorescent material with 5-HT-like characteristics was detected in P3 as well as in P2. This was confirmed in a subsequent more quantitative experiment. Figure 16 shows that P3 had fluorescent characteristics which corresponded to those of authentic 5-HT. Similar spectra were also obtained for P2. Furthermore the activation maximum occurred at 300 mμ, as for 5-HT. As a further test the solutions were placed in the light beam and exposed to the activating light (295 mμ) until the fluorescence had decayed to a stable value. The rate of decay of fluorescence was found to be similar to that observed for 5-HT. However, although the relative amounts of 5-HT in P2 and P3 were comparable by both methods of assay, the values obtained by the fluorescence method were about five times higher than those determined by biological

assay. The high values obtained by fluorescence-assay were probably due to high values for the reagent- and tissue-blanks. These blank values are of the order of 0.1 µg or more of 5-HT (Udenfriend, 1962; Boullin, 1963) and would be expected to introduce a large error into the estimation of small amounts of 5-HT such as were found in the particulate subcellular fractions after removal of the cytoplasmic fraction. In contrast, these small amounts of 5-HT were easily assayed with a reasonable degree of precision on the rat fundus, which was sensitive to about 0.00025 µg of 5-HT in an 8 ml bath and showed a good differentiation between doses. Thus it seems conclusive that the biological activity of P3 is due to 5-HT, although there may be other substances present tending to enhance the fluorescence of the low concentrations of 5-HT. The failure of Michaelson and Whittaker (1963) to find any 5-HT in this fraction is therefore unlikely to be due to a difference between biological and fluorimetric assay, and is more probably due to differences in the details of centrifugation.

To test the possibility that differences in the method of centrifugation could have accounted for the divergent findings regarding the localization of 5-HT in the microsomal fraction, some additional experiments were carried out in which the details of centrifugation were different from those in the basic method. There are two alternative explanations, based on differences in details of centrifugation, which, it was thought, could have accounted for the divergent findings. One explanation is that the 5-HT content of P3 is due to contamination by small nerve-ending particles which did not sediment with the bulk of the nerve-endings in P2. If this were so, then it is surprising that the ACh, determined in the same experiments did not have the same distribution,

since this would imply that the 5-HT-containing nerve-endings had quite different sedimentation-characteristics from those containing the ACh. An alternative explanation, which seems more likely (see Discussion), is that under certain conditions the P2 fraction is fairly heavily contaminated with microsomal particles, some of which contain the 5-HT. This would account for the higher RSA of the microsomal as compared with the crude mitochondrial fraction. Therefore, the modifications introduced into the centrifugation-technique were designed to yield information relating to these two possible explanations.

First of all, if the 5-HT in P3 was due to nerve-ending particles which had not been sedimented, it should have been possible to sediment these endings by recentrifuging the supernatant fluid obtained after sedimentation of the P2 particles. Secondly, if the second explanation was correct, then it might be possible to free the crude mitochondrial fraction of microsomal contamination by washing the P2 sediment more efficiently. Further information regarding the nature of the 5-HT-containing particles was obtained by centrifuging in progressively larger centrifugal fields.

Fig. 17 illustrates both the technique and the results which were obtained for ACh and 5-HT in two experiments.

The chief difference between the fractions on the left and on the right side of the figure is that the former were obtained after the P2 sediment was washed only once, whereas the latter fractions were obtained after washing the P2 sediment three times. The additional washing obviously had no effect on the distribution of either ACh or 5-HT. There was, however, a marked difference between the distribution of ACh and of 5-HT. All of the ACh was sedimented with the heaviest particles, which were

obtained in P2a and in P2c. In contrast, only 16 and 24 per cent respectively of the 5-HT was recovered from these particles. The remaining 5-HT was fairly evenly distributed between the particles obtained at later stages of the fractionation.

Now Michaelson and Whittaker (1963) sedimented their P2 fraction in a gravitational field of 940,000 g min. This would have sedimented all but the smallest microsomes (i.e. all except P3b and P3d) in the present study. If the 5-HT contents of P2a, P2b and P3a or P2c, P2d and P3c are pooled, then it may be seen that these fractions together contain 70 to 80 per cent of the particulate 5-HT, a result which is not so very different from that obtained by Michaelson and Whittaker (1963). However, the implications of this experiment are profound, since it shows quite conclusively that the 5-HT is contained in particles which are vastly different from the ACh containing particles, Furthermore, 5-HT was present in the smallest of these particles. This would suggest that the 5-HT is associated with microsomal particles in general, and that the small amount of 5-HT in the nerve-ending-particles is due to their content of microsomes.

f) Υ-Amino-n-butyric acid (GABA) and glutamic acid

The distribution of these two substances was investigated by method b) applied to guinea pig brain homogenates. Figure 15 illustrates the results obtained in two experiments in which the subcellular distributions of GABA, glutamic acid and ACh were determined. The distributions of GABA and glutamic acid were similar. About 70 per cent of the recovered amino acids was in the cytoplasmic fraction (S), in contrast with the low cytoplasmic content of ACh. Even in the presence of physostigmine only 30 per cent of the ACh was found in the cytoplasmic fraction.

However, there were additional small peaks in the distribution-curves for the amino acids in P2 and B_1.

The resemblance between the distribution of GABA, glutamic acid and potassium (Figs. 14 and 15) is quite striking. It was shown earlier that the distribution of potassium was adequately explained on the assumption that it was a freely diffusible substance, evenly distributed throughout the cytoplasm. The potassium content of P2 and its subfractions was slightly higher than was anticipated on this assumption, but could be accounted for in terms of the cytoplasmic content of these fractions. Similarly, the distribution of the amino acids, GABA and glutamic acid, suggests that they too are cytoplasmic constituents, which are not specifically localised in any subcellular particle, but are evenly distributed throughout the cytoplasm. The amounts of amino acids in P1, P3 and C_1 were virtually identical to the amounts expected from the theoretical distribution of a freely diffusible substance, but, as for potassium, the amounts in P2 and its subfractions were slightly higher. About 50 per cent of the amino acids in P2 were recovered from A_1, B_1 and C_1. The corresponding figure for potassium was 31 per cent. However, it was suggested that some of the potassium had diffused from the particles on subfractionation. Thus, the higher value for the amino acids may mean that they are not able to diffuse through the membranes surrounding the particles to the same extent as potassium.

That the amino acids are not specifically located in any particular fraction or subfraction is also borne out by an examination of the RSAs (Table 11), which showed little variation. This was in marked contrast to the RSAs for ACh and indicates that the amount of the amino acids present in each fraction was related to the quantity of nitrogen

present.

iii) Discussion

It was first shown by Hebb and Whittaker (1958) and Whittaker (1959) and confirmed by De Robertis et al., (1962), and in the present work, that the ACh-like substance in brain is located in a specific subcellular fraction, in which the major component consists of pinched-off nerve-endings (Gray and Whittaker, 1962; De Robertis et al., 1962). This cholinomimetic substance has been identified as acetylcholine by parallel biological assay and a combination of electrophoretic and chromatographic techniques (see section IV B). It is now well established that the ACh-content of this fraction is quite unrelated to the nitrogen content. However it could be argued that the amount of ACh is related to the volume of cytoplasm enclosed within the terminals, since in the presence of eserine about 30 per cent is recovered from the cytoplasmic fraction. This explanation assumes that the total volume of ACh-containing nerve-endings is much greater than that of the cell soma. This is considered to be unlikely since it has now been shown that potassium, which may be assumed to be an intracellular cytoplasmic component, and the amino acids GABA and glutamic acid, had quite different distributions, which were thought to reflect the relative amounts of cytoplasm in the various fractions. This conclusion is further substantiated by the observation (Johnson, 1960; Brunngraber, Aguilar and Occomy, 1963; Johnson and Whittaker, 1963) that certain glycolytic enzymes, thought to be of cytoplasmic origin, have a distribution similar to that found for potassium and the amino acids in the present experiments.

These observations, together with the recent finding that ACh is specifically localised in structures resembling synaptic vesicles

(De Robertis et al., 1963; Whittaker, Michaelson and Kirkland, 1963,1964) lend support to the now widely held belief that ACh serves some specific function in central synaptic transmission and they are difficult to reconcile with a more general role for ACh in axonal conduction (Nachmansohn, 1959).

It is clear that at peripheral cholinergic synapses acetylcholinesterase is closely restricted to the region of synaptic contact, either presynaptically, as in sympathetic ganglia, or postsynaptically, as at the skeletal neuro-muscular junction. In the C.N.S. the evidence derived from studies of the subcellular distribution is conflicting. Toschi (1959), Holmstedt and Toschi (1959) and Aldridge and Johnson (1959), found that acetylcholinesterase was localized in microsomes, whereas De Robertis et al., (1962) claim that it is closely associated with the synaptic endings, probably with the synaptic membranes, but certainly not with the vesicles (De Robertis, Rodriguez de Lorez Arnaiz, Salganicoff, Pellegrino de Iraldi and Zieher, 1963). De Robertis et al., (1962), attributed the discordant findings to a more extensive rupture of nerve terminals in the earlier experiments. Although it has been shown by histochemical methods that quite large amounts of cholinesterase are present in the cytoplasm of some neurones (Snell, 1961; Koelle, 1963), the enzyme is especially concentrated in the cell membrane (see Koelle, 1963): these results are in agreement with the distribution studies carried out by De Robertis et al., (1962). Thus the localization of acetylcholinesterase is in keeping with a transmitter function of ACh.

It seems to be agreed that choline-acetylase is concentrated in the nerve-endings (Hebb and Whittaker, 1958; Whittaker, 1959; De Robertis et al., 1963), but the evidence regarding the localization in

synaptic vesicles is irreconcilable: De Robertis et al., (1963) consider it to be concentrated in the vesicles, whereas Whittaker, Michaelson and Kirkland (1963, 1964) are extremely critical of the techniques used by De Robertis and co-workers, and obtained quite different results. They claim that it is not a component of the vesicles but is merely concentrated in the cytoplasm of the nerve endings. Whichever of these views is correct, the location of the enzyme which synthesizes the ACh seems to be admirably placed if the ACh were serving as a transmitter substance.

The similarities between the subcellular distributions of ACh and substance P suggest that the latter compound is also localised in nerve-ending-particles. Similar results for substance P in rabbit brain have also been obtained by Kataoka (1962a).

Recently Carlini and Green (1963a) have found in brain extracts a substance which produced slow contractions of the guinea pig ileum. This substance was soluble in acetone and therefore was assumed not to be substance P. Since it is not known whether the acetone-soluble substance was inactivated by chymotrypsin it could possibly have contributed to the estimates of substance P in the present work. It is unlikely however, that this would have been a serious source of error since the figures given by Carlini and Green demonstrate about a threefold reduction in activity of their acetone extracts as compared to their acid extracts. This indicates that most of the activity in acid extracts is due to substance P. Furthermore, their acetone extracts may have contained some substance P since it is known (Correale, 1957) that substance P from brain is more soluble in acetone than is substance P from intestine.

Although substance P has frequently been considered as a trans-

mitter substance within the mammalian central nervous system, there is no conclusive evidence to support this hypothesis (see Haefely and Hürlimann, 1962). However, crude substance P may contain a number of active polypeptides (Zetler, 1961) and one of these, so far unpurified, may be active within the central nervous system. In this connection, it is interesting to note that Krivoy and Kroeger (1963) have recently demonstrated that relatively small amounts of highly purified substance P, injected intravenously, has some effect on dorsal root potentials in decerebrate cats. However, the significance of this observation is not clear at the present time. It is also interesting to note that Mitchell and Ramwell (1963) have demonstrated the occasional release of an unidentified polypeptide from the surface of the cat's cerebral cortex, which could be substance P or one of its components, in addition to another substance which was not inactivated by chymotrypsin. However, it is also possible that substance P may have a carrier function in relation to ACh, although this has been considered to be unlikely (Crossland, 1957). Should more direct evidence for a transmitter function of substance P be found in the future, then the subcellular distribution of this agent would not be inconsistent with such a function.

A consideration of the literature on the subcellular distribution of 5-HT in brain reveals many anomalies. The first report appears to be that of Bogdanski, Weissbach and Udenfriend (1956) who found it to be equally distributed between the nuclear, mitochondrial and microsomal fractions. Similar results were obtained by Hughes, Kuntzman and Shore, (1957). Subsequently Walaszek and Abood (1957, 1959) found in their experiments that it was mainly located in the mitochondrial

fraction. This latter view is that held by Whittaker (1959), Michaelson and Whittaker (1963) and Michaelson, Whittaker, Laverty and Sharman, (1963) who showed that, on subfractionation of the crude mitochondrial fraction in a density-gradient, the 5-HT was located in a layer composed chiefly of synaptic terminals. It was claimed that the subcellular distribution was similar to that of acetylcholine. On attempting to confirm this observation it was found (Ryall, 1962a) that the microsomal fraction (P3) contained almost as much, or in some experiments, more 5-HT than the crude mitochondrial fraction (P2) and that the RSA of the microsomal fraction was consistently higher than that of the crude mitochondrial fraction. These findings have since been essentially confirmed by Zieher and De Robertis (1963) and Carlini and Green (1963a). However, subfractionation of P2 in a density-gradient yielded results comparable to those obtained by Michaelson and Whittaker (1963). A further anomaly exists in the finding by Kataoka (1962b) that the 5-HT in the crude mitochondrial fraction from rabbit brain was recovered from the most dense layer of the gradient, which contained free mitochondria. This last observation was also made in the present experiments when iproniazid was added to a guinea pig brain homogenate. However, in view of the demonstrated aggregating effect of iproniazid, it is felt that little significance can be placed on this result. It seems probable that similar effects could have occurred in Kataoka's experiments, since in a later paper (Inouye, Kataoka and Shinagawa, 1963) an aggregation of particles in their dense subfractions was noted, but was not commented upon.

How then can these anomalies be explained? In particular what is the explanation of the difference between the results obtained for 5-HT in the

present experiments and those of Whittaker (1959) and Michaelson and Whittaker (1963) although results obtained for acetylcholine are very similar. Kataoka (1962b) criticized Whittaker's earlier results, which were obtained by biological assay, on the ground that precautions had not been taken to inactivate substance P. However, Whittaker (1962) has refuted this suggestion and Michaelson and Whittaker (1963) confirmed the earlier findings by means of fluorimetric assay. Fluorimetric assay tends to give higher values for 5-HT than biological assay (Carlini and Green, 1963a) and may become unreliable when attempting to assay small amounts (Udenfriend, 1962; Boullin, 1963). This is not likely to be the explanation of the divergent findings since by biological and fluorimetric assay, on the same extracts, in the present investigation the relative amounts of 5-HT in the crude mitochondrial and microsomal fractions were similar, although fluorescence-assay gave higher absolute values. A more likely cause of the discrepancies may lie in the different centrifugal fields used to separate the microsomal from the crude mitochondrial fractions. It is extremely difficult to free mitochondrial particles from microsomal contamination, although microsomes can be obtained in a relatively pure form (Hogeboom, Schneider and Palade, 1948; Palade and Siekevitz, 1956; Toschi, 1959). Many washings of the mitochondrial sediments are necessary to achieve a good separation. Thus relatively high centrifugal fields, such as those used by Whittaker and his co-workers, may increase the risk of microsomal contamination of P2 (see also Whittaker, Michaelson and Kirkland, 1963). Since the RSA of the microsomal fraction was higher than that of the crude mitochondrial fraction, it therefore seems possible that the 5-HT content of the latter was partly due to microsomal

contamination.

Some evidence in support of this suggestion was obtained when particles, smaller than those which sediment in the nuclear fraction, were carefully sedimented in progressively higher centrifugal fields, with adequate washing of the particles. By this technique it was possible to achieve an almost complete separation of the particles which contained the ACh from those which contained the 5-HT. In contrast with ACh, the 5-HT was fairly evenly distributed among all of the fractions, down to the smallest of the microsomes. These particles were not synaptic vesicles since both Whittaker, Michaelson and Kirkland (1963) and Zieher and De Robertis (1963) are agreed that ACh, but not 5-HT, is present in synaptic vesicles. Whittaker (personal communication) has evidence that microsomal material is contaminated with small nerve-endings, unless the crude mitochondrial fraction is sedimented in the very high centrifugal fields such as he and his co-workers used. However, it seems unlikely that these nerve-endings would have been sedimented along with the smallest of the microsomes. Thus the most reasonable explanation of the results is that 5-HT is a microsomal component, and that the 5-HT content of nerve-endings is due to their content of microsomes.

The microsomes are thought to be derived from the endoplasmic reticulum (Palade and Siekevitz, 1956; Palay and Palade, 1955; Hanzon and Toschi, 1959; Toschi, 1959). Thus 5-HT would seem to be associated with the endoplasmic reticulum, rather than specifically with synaptic structures, and on this basis the 5-HT in the nerve-endings could be due to their structural integrity and their content of endoplasmic reticulum. However, until more is known about the function of this

system, the function of 5-HT remains obscure and it may be concluded that, since it does not appear to satisfy criterion 1 (see page 20), it is unlikely to be a synaptic transmitter.

It remains to ascertain the degree of correspondence between this interpretation and the results obtained by another recently developed method, namely the fluorescence-technique which has been applied to the cellular visualization of 5-HT in the central nervous system by Falck and his co-workers (Carlsson, Falck and Hillarp, 1962; Bertler, Falck and Owman, 1963; Carlsson, Falck, Fuxe and Hillarp, 1964; Owman, 1963, 1964). These workers have demonstrated the presence of material with a fluorescence similar to that of 5-HT. However, the visualization of 5-HT was not usually good enough to determine the cellular location of the amine. They advanced two explanations: the first was that the 5-HT was localized in structures that were insufficiently packed to reach the limit of detection, and the second was that 5-HT was so widespread that at any particular site the concentration again did not reach the limit of detection. The second of these interpretations is compatible with the present results derived from a study of the subcellular distribution in brain homogenates. Furthermore, it was shown that at least half of the 5-HT in the pineal gland was not associated with nervous structures (Bertler, Falck and Owman, 1963; Owman, 1963). Other evidence concerning the location of 5-HT in the medial geniculate body has been obtained by Utley (1963). He showed, by denervation experiments, that most of the 5-HT was probably associated with glial elements. Thus all of this evidence is consistent with the suggestion that 5-HT is not a transmitter in the mammalian central nervous system.

There is a close relationship between the subcellular distribution of the amino acids, GABA and glutamic acid, and of intracellular potassium. Both the amino acids and potassium were chiefly located in the cytoplasmic fraction. However, a small proportion was present in the nerve-ending fraction, and this could be attributed to the volume of cytoplasm trapped within the nerve-endings. Since the preliminary report of these findings (Ryall, 1962b), similar results for GABA in mouse brain homogenates have been obtained by Weinstein, Roberts and Kakefuda (1963), who used somewhat different techniques. They interpreted their results in a different fashion and concluded that GABA was associated with presynaptic fractions. However they did not consider the possibility that the small amount of GABA in the nerve-endings could be due to their cytoplasmic contents. The present results suggest that GABA and glutamic acid may be cytoplasmic constituents distributed evenly throughout the neurone.

There are some authors who consider that GABA or one of its derivatives, in particular, β-hydroxy-γ-aminobutyric acid, may be an inhibitory transmitter in the nervous system (Hayashi, 1958, 1960; Kuriaki, Yakushiji, Noro, Shimizu and Saji, 1958; Hayashi, Takashita, Namba and Kubo, 1962; Florey, 1964; Krnjevic, Randic and Straughan, 1964). However, the evidence for the occurrence of β-hydroxy-γ-aminobutyric acid is unconvincing (Mitoma, 1960; Pisano, Wilson and Udenfriend, 1960). More recently, Eccles, Schmidt and Willis (1963a) have suggested that GABA may be the transmitter producing the phenomenom of presynaptic inhibition, and Krnjevic et al., (1964) have proposed glutamic acid as an excitatory transmitter. On other evidence, these suggestions seem to be extremely unlikely (see Sections IV E, and

IV F). The present evidence, from studies of the subcellular distribution of amino acids, is consistent with the view that they do not function as synaptic transmitters in the mammalian C.N.S.

In addition to the substances already discussed, the subcellular distribution of nor-adrenaline (Chrusciel, 1960; Inouye, Kataoka and Shinagawa, 1963; Laverty, Michaelson, Sharman and Whittaker, 1963; Michaelson, Whittaker, Laverty and Sharman, 1963; Potter and Axelrod, 1963), and of histamine (Michaelson and Whittaker, 1962; Carlini and Green, 1963a,b; Michaelson and Dowe, 1963) have also been studied by techniques similar to those used in the present investigation. For both substances there is conflicting evidence. Laverty et al., (1963) and Michaelson et al., (1963) showed that the distribution of nor-adrenaline was similar to that of 5-HT, except that there was rather more nor-adrenaline than 5-HT in the microsomal fraction; they claimed that both nor-adrenaline and 5-HT were associated with nerve-endings, but not with synaptic vesicles. However, Inouye et al., (1963) showed, in their experiments, that both 5-HT and nor-adrenaline were present in particles more dense than nerve-endings. The reasons for doubting the results obtained by Inouye et al., (1963) have already been presented in the discussion on 5-HT. In that section an alternative explanation of the results obtained by Whittaker and his co-workers was advanced and a rather different interpretation of the data was made. If the subcellular location of nor-adrenaline is in fact similar to that of 5-HT, then the same interpretation as for the latter compound will suffice. Falck and his associates (see review by Carlsson, Falck and Hillarp, 1962 and Carlsson, Falck, Fuxe and

Hillarp, 1964) have studied the histochemical location of nor-adrenaline in the brain and spinal cord by their fluorescence-method. Their results clearly showed that some, at least, of the nor-adrenaline was closely associated with nerve-fibres and perikarya, but the method does not really allow an analysis to be made of the precise subcellular location of the amines and the apparent concentration in the varicosities which they describe may be due to the greater volume of the swellings compared with the intervening lengths of the nerve-fibres.

The most reliable results on the distribution of histamine in brain homogenates seem to be those obtained by Carlini and Green (1963a,b). They showed that histamine was mainly associated with microsomal elements. These workers used a biological method for the assay of histamine and demonstrated that fluorescence-assay, such as Laverty et al., (1963) and Michaelson et al., (1963) used was quite inadequate for the estimation of histamine in brain: there were at least five substances in brain which produced a fluorescence similar to that of histamine.

We are now in a position to review briefly some of the evidence relating to the subcellular disposition of substances which could possibly serve as transmitters in the central nervous system.

It seems to be generally agreed that acetylcholine, choline-acetylase and probably acetylcholine esterase are especially concentrated in the synaptic regions of neurones in the C.N.S. Furthermore, ACh (and possibly choline-acetylase) is localized in synaptic vesicles; this provides the first direct evidence that the vesicles do in fact contain the transmitters. When this is taken in conjunction with the large body of evidence supporting a transmitter function for ACh in

the C.N.S., the evidence seems incontrovertible. On the other hand, although the subcellular distribution of substance P is suggestive of a transmitter function, there is not yet any direct evidence to support such a contention: only one of the criteria (criteria 1, see page 20) has been satisfied.

In the distribution of GABA and glutamic acid we have fairly conclusive evidence that they are cytoplasmic constituents, spread evenly throughout the neurone. This provides no evidence in favour of a transmitter function and suggests that, if they do have a role to play in regulating neuronal activity, it is by an intracellular metabolic mechanism.

I have also discussed the evidence relating to the cellular location of 5-HT, nor-adrenaline and histamine. It is the author's view that 5-HT and histamine are distributed in similar manner and are not specifically associated with nerve-terminals. Instead, they seem to be associated with particles of microsomal dimensions, and the amounts of these substances in the terminals could be due to their microsomal contents. Since the microsomes are considered to be derived from the endoplasmic reticulum, the data suggest that their functions are in some way related to the role which this structure plays in the regulation of neuronal function. However, this role has by no means been elucidated. The available evidence concerning the distribution of nor-adrenaline suggests that its distribution is similar to that of 5-HT, and again it is concluded that the distribution provides little support for the view that it is a synaptic transmitter.

Finally, a word of caution must be introduced into this discussion. Obviously, when studying the distribution of a substance within a

tissue, we are dealing with the total amount of the substance in that tissue, and the possibility exists that the substance in question may be subserving a number of functions. Thus the overall distribution may not adequately reflect the distribution of a small amount, which is serving a minor function. Unless the evidence for such a minor function is overwhelmingly strong, then, in order to economize on hypotheses, the simplest interpretation consistent with the facts should be taken.

D) The action of brain extracts on central neurones

In the past, the search for biologically active substances in brain extracts has been largely confined to testing the actions of extracts on isolated tissues, or on physiological systems not directly concerned with the transmission of nervous impulses in the C.N.S. This led to the important discoveries of substances such as ACh, nor-adrenaline, histamine and 5-HT in brain. When searching for unknown transmitters, such techniques have the limitation that they do not yield information about the actions of a substance at its site of origin, i.e. in the C.N.S. This limitation may be partially overcome by testing the effects of the extracts on central nervous processes by either topical or systemic administration. For example Crossland (1957), Crossland, Garven and Mitchell (1959) and Crossland and Mitchell (1956) found that extracts of the cerebellum, when injected into the carotid artery, led to an increase in the electrical excitability of the cerebellum, and that a cerebellar enzyme inactivated this substance. The activity of the extract could not be attributed to the actions of any known substance; the active material which has still to be identified, was quite ineffective on a wide variety of isolated organ-preparations. However, the results obtained by such studies may be influenced by indirect effects such as local vascular changes, or modifications of the transport of necessary metabolic factors to the neurones. Further defects are that the access of the injected substance may be hampered by the interposition of the 'blood-brain-barrier' between the neurones and the circulation, and the site

of action of the injected material is not accurately localized. In these respects a technical improvement is the administration by topical application of the extract to exposed nervous tissue. Thus McLennan and his co-workers (see review by McLennan, 1962) have applied Factor I extracts from brain to the spinal cord and obtained a depression of monosynaptic reflexes which was blocked by strychnine. Topical application suffers from the disadvantage that the degree of penetration of active material into the neuronal tissue cannot be ascertained and the site of action is still not accurately localized.

It seemed that many of these objections could be overcome, and more direct evidence of transmitter-like actions obtained, if it were possible to apply brain extracts directly into the extra-neuronal environment, while at the same time recording the electrical activity of a neurone close to the point of ejection. The method of electrophoretic ejection of compounds from multi-barrelled micropipettes, coupled with unitary recording from the neurones under study, appeared to meet these requirements.

If an electric current is passed through a solution containing a mixture of ions, then the amount of charge carried by any one ion is determined both by its concentration, relative to that of the other ions present, and by its transport number or intrinsic electrophoretic mobility. Now, a brain extract contains several species of ions, some biologically active and some inactive. The problem is to obtain highly purified brain fractions, so that active ions in the fractions would carry a sufficiently large proportion of the electrophoretic current and so be ejected in quantities adequate to affect the nearby neurones.

It is generally believed that substances which function as transmitters are especially concentrated in the nerve-terminals. Thus one method of reducing the proportion of unwanted ions in the extract was to carry out a preliminary fractionation of the brain homogenate, before subjecting the extract to electrophoretic and chromatographic purification. Therefore, the crude mitochondrial fraction from brain was used in this study. Since sucrose, in high concentrations, interfered with the electrophoretic and chromatographic separations, the sucrose content of P2 was reduced by resuspension of the particles in NaCl, followed by resuspension in water to lower the NaCl concentration (see section III B). The P2, rather than the nerve-terminal-fraction, was employed since some loss of ACh occurred when the P2 fraction was further subfractionated (see section IV C). However, the ACh content of P2 accounts for most of the ACh in brain, and in obtaining this fraction, the amounts of cytoplasmic substances and of substances concentrated in parts of the neurone other than the terminals, are greatly reduced. Subsequently, the extract from the P2 fraction was separated into cations and anions by paper-electrophoresis (see section III B). Thus the cations and anions were subjected to paper-chromatography separately. As a consequence of this separation, the electrophoretic ejection of ions derived from the brain extract could be controlled by suitably directed currents through the micro-electrode.

The method by which the various fractions were obtained by electrophoresis and chromatography is described in section III B, and the distribution of activity on several isolated organ-preparations is discussed in section IV B.

It was necessary to establish that the methods were adequate to demonstrate the presence of a substance with known effects on central neurones. It was known that certain of the extracts contained ACh (see section IV B). Therefore, the action of the ACh-containing fractions was determined on Renshaw cells in the spinal cord of anaesthetised cats (see section III E). These neurones are very sensitive to the action of electrophoretically administered ACh, and so were admirable test-objects for the purpose. Furthermore, there is good evidence that ACh is a transmitter at this site (see section V). If it could be shown that ACh was present in the brain extract in sufficient concentration, and at a sufficiently high state of purity, to be detected by its action on Renshaw cells, there was a reasonable chance that other unidentified transmitters would be detected by their actions on appropriate neurones.

Fig. 18 illustrates the action of the ACh-containing fraction (peak 2, Fig. 4 B_1) on a single Renshaw cell. The extracted ACh was estimated to contain about 10 µg of ACh (as the bromide). This was dissolved in water to give a final concentration of 2 mM with respect to the ACh, but there were obviously other ions present since the conductivity of the solution was similar to that of a 165 mM solution of NaCl. The solution was placed in one barrel of a five-barrelled micro-electrode of overall tip diameter 5 µ. The three other outer barrels contained 2 mM ACh bromide (in 165 mM NaCl), edrophonium chloride (0.1 M) and tetraethylammonium bromide (0.1 M). Extracellular spike potentials were recorded through the centre barrel which was filled with 4 M NaCl, and the frequency of firing was continuously displayed on a paper-recorder, as described in Methods (Section III E).

The cells were usually fired when ACh was passed from a 2 mM solution by a current of 10 to 15 nA. Slightly larger currents were required to excite the cells with the ACh from the extract, which produced a lower firing frequency than was obtained with authentic ACh (Figs. 18 and 19). This difference is attributable to an interfering cationic substance in the extracts, which partially blocked the action of ACh, applied simultaneously, and this is illustrated in Fig. 19. The blocking action was also obtained with other eluates. Fig. 20 shows the blocking action of a substance eluted from a chromatogram with triethylamine buffer (E) and of triethylamine itself (B), on cortical neurones and dorsal horn cells, in which firing was induced by the electrophoretic application of DL-homocysteic acid, and on a Renshaw cell which was also fired by ACh. Thus the blocking action originates from the chromatographic paper and from solvents used in the purification of the extracts, and is thought not to be of biological origin.

In the presence of edrophonium (Fig. 18) the excitant effect of both the extracted and authentic ACh on Renshaw cells was augmented. Tetraethylammonium (Fig. 18) and intravenously injected dihydro-β-erythroidine (0.1 - 0.2 mg/kg) blocked the excitant actions of both to a similar degree.

Thus it has been shown that there is a substance present in a purified extract, obtained from a fraction of a brain homogenate containing synaptic terminals, which had an action indistinguishable from that of authentic ACh on Renshaw cells. From this it is concluded that the techniques employed might be sufficiently sensitive to detect the

presence of other active compounds which could be present in the purified extracts, although it was expected that the results would be complicated by depressant effects due to substances of non-biological origin. Therefore it was decided that the remaining fractions, which did not contain ACh, should be examined for excitant or depressant effects on single neurones.

Since there was a large number of samples to be examined, the effects of these samples were tested on cortical neurones. Several such neurones could be found on a single penetration of the micro-electrode and it was possible to test each fraction on at least five cells: no attempt was made to identify these cells. A few extracts were also tested on spinal interneurones.

A number of difficulties arose during these experiments. First, the electrical resistance of many of the micropipettes which contained extracts increased when currents were passed through them, and the maximum currents were limited to about 10-20 nA. Thus, even if active compounds had been present, they may not have been passed out in sufficient quantity to affect the nearby neurones. Secondly, cationic substances in most of the eluates from paper chromatograms of brain fractions, which were eluted with either pyridine or triethylamine-containing buffers, had effects on the cells indistinguishable from those of the organic bases which the buffers contained (Fig. 20). In addition, eluates from blank papers had similar effects. In no case was the action of an eluate from a chromatogram greater than that of the triethylamine which was passed out of the electrode with the same current. On Renshaw cells, the organic base had a greater effect on

the responses to ACh than it had on the responses to an excitant amino acid (Fig. 20). Thus, the only conclusion to be drawn from these experiments is that chromatograms of the cationic constituents of a crude mitochondrial fraction from brain contained no highly active components other than ACh and an unidentified compound, probably formed by the reaction between the organic base in the buffered solvents and anionic constituents of the paper. When testing anionic constituents of the extracts this difficulty did not arise, but no evidence was obtained that the extracts contained anionic components capable of affecting neurones. The failure to detect either aspartic or glutamic acids, which are excitants of neurones (Curtis, Phillis and Watkins, 1960), was not unexpected since the content of glutamic acid in the crude mitochondrial fraction is very low (see section IV C).

In conclusion, the techniques were adequate to demonstrate the presence of ACh in a brain extract, by its effect on central neurones, but they were inadequate to reveal the presence of other active cations or anions. Further progress along these lines will have to await the development of more refined methods of purification in which the introduction of active contaminants from extraneuronal sources is avoided. When such techniques have been developed it may prove to be possible to purify larger quantities of extract and so permit the detection of compounds of a lower order of activity.

E) The action of drugs on presynaptic terminals

i) Introduction

Eccles (1964) has recently reviewed the subject of presynaptic inhibition in the central nervous system. Stimulation of appropriate afferent fibres diminishes spinal reflexes and reduces the size of intra-cellularly recorded excitatory postsynaptic potentials, and yet there is no associated change in the postsynaptic membrane, neither in its resting potential, nor in its electrical conductance, nor in its electrical excitability (Frank and Fuortes, 1957; Frank, 1959; Eccles, 1964). It has therefore been postulated that this type of inhibition is effected by a reduction in the amount of transmitter released at excitatory terminals, rather than by the postsynaptic action of an inhibitory transmitter (postsynaptic inhibition). The early investigations of Gasser and Graham (1933) and of Barron and Matthews (1938) established that dorsal root volleys produced slow potential changes within the spinal cord and that these changes were associated with an electrotonically propagated depolarization of dorsal root fibres. More recent studies (Eccles, Eccles and Magni, 1961; Eccles, Magni and Willis, 1962; Eccles, Schmidt and Willis, 1962, 1963b,c) have established a causal relationship between presynaptic inhibition and the depolarization of primary afferent fibres. There are many consequences of this depolarization: The excitability of the primary afferent fibres is increased (see Eccles, Magni and Willis, 1962; Eccles, Schmidt and Willis, 1963b; Eccles, 1964), slow potentials are recorded from the dorsal surface of the cord (Gasser and Graham, 1933), the size of the action potentials propagating towards the excitatory synaptic terminals is decreased

(Eccles, Schmidt and Willis, 1963c; Eccles, 1964), which presumably reduces the amount of transmitter released by each impulse (see Eccles, 1964).

The primary afferent depolarization is considered to be the consequence of the depolarizing action of a transmitter at axo-axonic synapses upon afferent terminals (see Eccles, 1964). Thus the term presynaptic inhibition refers to the overall result and not to the mechanism by which transmitter release is diminished.

Since presynaptic inhibition involves the action of transmitter substances, it is of interest to compare the pharmacology of presynaptic inhibition with that of postsynaptic inhibition. Such studies may eventually yield clues to the nature of the respective transmitters.

The pharmacology of presynaptic inhibition in the mammalian nervous system differs from that of postsynaptic inhibition. Whereas postsynaptic inhibition in the spinal cord is blocked by strychnine, presynaptic inhibition is unaffected, but it is reduced by picrotoxin, which has no effect on postsynaptic inhibition (see Eccles, 1964). These and other pharmacological observations have given rise to the suggestion that the transmitter involved in presynaptic inhibition is not the same as that which operates in postsynaptic inhibition (Eccles, Schmidt and Willis, 1963a; Schmidt, 1963). However there are now a number of authentic cases of strychnine resistant postsynaptic inhibition (see Andersen, Eccles, Loyning and Voorhoeve, 1963; Crawford, Curtis, Voorhoeve and Wilson, 1963a) and the variations in the pharmacological affinities of the inhibitory receptors may merely

reflect minor differences in the receptors, such as are thought to exist with nicotinic and muscarinic cholinoceptive receptors, rather than differences in the transmitters which produce the two types of inhibition.

A more specific suggestion regarding the identity of the presynaptic inhibitory transmitter was made by Eccles, Schmidt and Willis (1963a). They found that both γ-amino-n-butyric acid (GABA) and 3-amino-1-propanesulphonic acid depressed the dorsal root potentials and increased the dorsal root reflexes when the amino acids were applied to the surface of the feline spinal cord. Moreover, Schmidt (1963) showed that these amino acids and glutamic acid produced an increase in the excitability of primary afferent fibres in the toad spinal cord. These results led Eccles, Schmidt and Willis (1963a) to suggest tentatively that GABA or a closely related compound could be the depolarizing transmitter acting at the presynaptic synapses. However, Schmidt (1963) suggested that part of the depression of the dorsal root potential produced by GABA and 3-amino-1-propanesulphonic acid was due to a depression of the interneurones on the presynaptic pathway.

The results obtained by Eccles, Schmidt and Willis (1963a) and Schmidt (1963) could have been produced by an effect of the amino acids at sites other than the subsynaptic receptors on the terminals of primary afferent fibres, since the method of applying the solutions to the spinal cord did not allow a precise analysis to be made of the actual locus of action. Therefore the present experiments were carried out in an attempt to overcome this objection, and to determine more

precisely the action of amino acids on the excitability of presynaptic terminals known to be depolarized by impulses in appropriate presynaptic inhibitory pathways.

ii) Results

A general description of the apparatus and of the technique is given in section III E. The object of the experiments was to determine the excitability of the primary afferent fibres close to their terminals before, during and after the micro-electrophoretic application of amino acids and other substances in the immediate vicinity of the terminals.

Although glutamate ion in relatively high concentrations ($5\text{-}50 \times 10^{-3}$ M) depolarizes frog motor nerve fibres (Sasaki, 1958) it has no effect on mammalian axons in the spinal cord when it is passed electrophoretically into the vicinity of axons which have been penetrated by a microelectrode (Curtis, personal communication). GABA in very high concentrations (0.05-0.5 M) has no effect on the propagation of impulses in mammalian dorsal or ventral root fibres (McLennan, 1957; Curtis, Phillis and Watkins, 1959). Thus mammalian nerve fibres appear to be relatively insensitive to the actions of amino acids. It was therefore assumed that the changes in the excitability of primary afferent fibres produced by electrophoretic ejections of amino acids in the vicinity of the terminals were due to an action on the terminals or possibly on the adjacent non-myelinated preterminal segment of the axon.

One of the major difficulties encountered was to orientate the micro-electrode accurately with respect to the terminals. There

were several ways in which this was achieved. First, the five-barrelled micro-electrode was inserted into the dorsal or ventral horn of the spinal cord while recording through the centre barrel the monosynaptic extra-cellular field potentials. These potentials were generated by stimulation of the group Ia muscle afferent or low threshold cutaneous afferent nerves in the hind limb. When these potentials reached their maximum size, it was reasonable to assume that the tip of the electrode was in a region where there was a large number of neurones fired monosynaptically and a correspondingly high density of nerve-terminals impinging upon them. In order to ascertain that sufficient amino acid could be ejected from the electrode to act on the synapses, the depressant effect of electrophoretically applied GABA was tested on the postsynaptic field potentials (Curtis, Phillis and Watkins, 1959). If no effect was obtained, then the electrode was moved slightly until a reduction in field potentials occurred. Sometimes a small presynaptic spike could be seen preceding the field potential generated by the cells and this aided the placement of the electrode. The centre barrel of the electrode was then connected into the stimulating circuit, and the electrodes on the peripheral nerves and the ventral roots were connected to the recording amplifiers. Graded stimuli were applied through the micro-electrode and the antidromic action potentials set up in the primary afferent fibres were observed on an oscilloscope and several superimposed traces were photographed.

When investigating the effect of drugs on the excitability of primary afferent fibres with monosynaptic connections onto motoneurones,

the excitability of the motoneurones was also observed by photographing the short latency (< 0.2 msec) responses set up in the ventral roots. Very small movements of the electrode were found to cause a large change in the excitability curves. It was therefore necessary to carry out several control runs over a number of minutes in order to be sure that conditions were relatively stable. If the excitability curve did not recover after the administration of a drug, the results were ignored since the result could have been produced by movement of the electrode.

Effects were not due to current alone since the ejection of sodium ions by even larger currents did not have any significant action on the excitability of muscle afferent or cutaneous afferent fibres.

It is probable that responses detected at low stimulus intensities were due to the stimulation of terminals close to the tip of the micro-electrode, whereas terminals further away were stimulated only by stronger stimuli. However, some of responses detected with small stimuli may have been due to the stimulation of fibres en passant. The failure in some experiments (e.g. Fig. 22) to change the threshold with drugs which either increased or decreased the response obtained with stronger stimuli may be due to the inability of these drugs to act on the shafts of the fibres.

The action of the local anaesthetic, procaine, was of interest since it demonstrated that drugs could be ejected from the micro-electrode in sufficient concentration to reduce the excitability of fibres which were activated by stimuli passed through the same electrode. Fig. 21 illustrates a typical effect of procaine on the excitability

of group Ia fibres in the flexor digitorum longus (FDL) nerve when the terminals were stimulated by means of a micro-electrode placed in the FDL nucleus in the ventral horn. This reduction in the excitability of primary afferent fibres was accompanied by a reduction in the excitability of the motoneuronal soma. Procaine was also observed to decrease the excitability of cutaneous afferent fibres.

In the majority of experiments in which GABA was used, the amino acid was dissolved in water to give a concentration of 2 M and the pH was then adjusted to pH 3-4 with HCl in order to ensure maximal ionization as a cation. The action of GABA (Fig. 22) was similar to that of procaine, but the effects were less marked. Sometimes an additional effect was observed. At high intensities of stimulation the excitability curve was lowered, but the threshold stimulus required to evoke a response was decreased. Although the upper part of the curve recovered with a time course similar to that of the extracellular field potentials, the threshold did not recover over relatively prolonged periods and there was occasionally a further reduction in the threshold when the GABA ejection ceased (Fig. 23). It was suspected that the reduction in threshold may have been due to damage to nerve terminals and fibres in the immediate vicinity of the micro-electrode.

When GABA is passed from a solution of about pH 3 as a cation, each ion would be expected to release a proton when it reaches the extracellular fluid, and so produce a reduction in local pH (Curtis and Watkins, 1960a). Since hydrogen ions affect the activity of neurones (Curtis, Phillis and Watkins, 1959; Curtis and Watkins, 1960), it was thought that the prolonged increase in the excitability of terminals

which were activated at low stimulus intensities, and were presumably close to the micro-electrode, may have been due to local changes in pH brought about by the electrophoretic ejection of GABA from solutions of pH 3-4. The effect of the electrophoretic ejection of hydrogen ion from 0.1 M solutions of HCl was therefore investigated. Sometimes it was ineffective but when an effect was observed it was similar to that produced by GABA passed from solutions of low pH (Fig. 24). There was sometimes a depression of the excitability of the fibres activated at suprathreshold stimulus strengths, and a reduction in the threshold stimulus. At other times the only effect observed was a reduction in the threshold stimulus. However, the depression of the excitability curves by GABA seemed to be greater than that produced by hydrogen ions.

The depressant action of GABA on primary afferent fibres was confirmed when the amino acid was passed by cationic currents from solutions of pH 5-6. At this pH GABA is mainly in the zwitterionic form (pKa_1 = 4.23), and thus would not be expected to release a proton when it reaches the extracellular fluid; the amino acid presumably passes from the electrode predominantly by electro-osmosis. When GABA was passed from such solutions (Fig. 25), there was a marked and fully reversible reduction in the excitability both of the primary afferent fibres of the biceps semitendinosus (BST) nerve and of the somas of the BST neurones, as shown by a reduction in the short latency responses recorded from the ventral root.

The action of acidic amino acids was more consistent. Since these were ejected as anions from solutions which were in the region of neutrality, the pH of the extracellular fluid would not be expected to

alter (Curtis and Watkins, 1960a). Two 'excitant' acidic amino acids were tested. DL-homocysteic acid (DLH), is a more powerful excitant of neurones than L-glutamic acid (L-GLUT) (Curtis and Watkins, 1963) and had a similar effect to L-GLUT on the excitability of primary afferent fibres, but was possibly slightly more active. The graph in Fig. 26 shows the excitability curves obtained for gastrocnemius group Ia fibres before, during and after the electrophoretic ejection of DLH with a current of 100 nA around the terminals of these fibres in the gastrocnemius nucleus in the ventral horn. The excitability of the terminals activated by the intermediate stimulus intensities was increased, but there was less effect at higher stimulus intensities, possibly because the terminals excited by these stronger stimuli were too far away from the electrode to be accessible to the amino acid.

In addition to testing the actions of amino acids on the excitability of primary afferent fibres, a few experiments were also carried out to examine the actions of carbamylcholine, nor-adrenaline, picrotoxin and strychnine. In five tests no effect was obtained with carbamylcholine ejected with currents up to 70 nA, and in three tests ejection of picrotoxin by currents up to 100 nA had no effect. In four tests with nor-adrenaline there was no effect in two, and a slight increase in excitability in the other two. The increase in excitability with nor-adrenaline was so slight that it could have been due to a small movement of the electrode with respect to the terminals under test. Strychnine was tested on seven occasions and each time there was a reduction in the excitability of the primary afferent fibres; in some experiments the reduction was more marked than in others. The action of strychnine on gastrocnemius group Ia fibres is illustrated in Fig. 27. In this experiment the shape of the control

excitability curve was typical of that observed in most experiments (see also Figs. 21, 22, 23, 24, 25 and 26). As the stimulus strength was increased, there was a stepwise increase in the size of the response recorded from the peripheral nerve. These steps presumably indicate either the heterogeneous nature of the population of terminals which are being stimulated or the fact that the terminals or fibres tend to be aggregated in groups at certain finite distances from the micro-electrode. Perhaps this grouping reflects the fact that the electrode was nearer to one neurone than to others in the same field, and therefore the terminals associated with that neurone would be more easily excited by electrical pulses than would terminals situated on neurones at greater distances from the micro-electrode. In this way it is possible to explain the observation that when the excitability of the terminals was depressed by drugs, the excitability curve was no longer parallel to the control curve, but tended to alternately approach it and diverge from it.

iii) Discussion

The experiments have shown that GABA depresses and that the acidic amino acids (DL-homocysteic and L-glutamic acid) increase the excitability of primary afferent fibres in the spinal cord of the anaesthetized cat.

GABA has no effect on crustacean nerve fibres (Kuffler and Edwards, 1958) or on mammalian nerve fibres (McLennan, 1957; Curtis, Phillis and Watkins, 1959; Curtis, personal communication), and glutamic acid has no effect on crustacean nerve fibres (Van Harreveld, 1959), although

at very high concentrations it depolarizes amphibian fibres (Sasaki, 1958). Thus it seems likely that the effects observed in the present experiments were due to an action on non-myelinated terminals or pre-terminal segments of the primary afferent fibres. These effects are consistent with the actions of amino acids on the soma-membrane of all types of nerve cells which have so far been investigated (Curtis, Phillis and Watkins, 1959, 1960, 1961; Curtis and Watkins, 1960a,b, 1963; Krnjevic and Phillis, 1961, 1963a,b; Crawford, Curtis, Voorhoeve and Wilson, 1963b; Andersen and Curtis, 1964a; Crawford and Curtis, 1964).

Although Kuffler and Edwards (1958) consider that the action of GABA on the crustacean stretch receptor is not localized to sub-synaptic areas, but may also be exerted on the soma-dendritic portions of the membrane which have no inhibitory synapses upon them, Curtis (1961 and personal communication) considers the actions of amino acids on mammalian central neurones to be confined to subsynaptic areas. The evidence for this latter view is not strong and it seems impossible to exclude, on present evidence, an action on extrasynaptic portions of the neuronal membrane. However, Diamond (1963) has shown that only certain regions, close to inhibitory synapses, of the membrane of the Mauthner neurone in the goldfish were sensitive to GABA, whereas the whole of the membrane was sensitive to glutamic acid. In view of this somewhat conflicting evidence, it seems unjustifiable to conclude that the effects on terminals observed in the present experiments were confined to those areas of the membrane immediately underlying the axo-axonic synapses. These observations on terminals provide no support

for the suggestion that GABA may be the transmitter which depolarizes the terminals of primary afferent fibres during the presynaptic inhibitory process (Eccles, Schmidt and Willis, 1963a). The depression of the dorsal root potentials when GABA was applied to the dorsal surface of the spinal cord (Eccles et al., 1963a) was probably due to the depressant effect of GABA on transmission at interneuronal junctions on the presynaptic depolarizing inhibitory pathway. There is no satisfactory explanation of the increase of excitability of dorsal root fibres in the toad spinal cord when GABA was applied to the surface (Schmidt, 1963) or of the increased dorsal root reflexes (Eccles et al., 1963a). However, GABA may reduce the background inhibition on the presynaptic pathway, so causing facilitation: the increase in presynaptic inhibition produced by strychnine has also been attributed to the removal of background inhibition (Eccles et al., 1963a). The many possible sites at which GABA may act on the pathway for presynaptic inhibition may explain the variable effects which have been observed (Eccles et al., 1963a).

The ability of glutamic acid to increase the excitability of afferent terminals is consistent with the hypothesis that an acidic amino acid may be the transmitter involved in presynaptic inhibition. However, an increase in excitability is characteristic of the action of acidic amino acids on many types of central neurones (loc. cit.), and at these sites the effect has been considered to be non-specific and unrelated to transmitter action (Curtis, Phillis and Watkins, 1960; Curtis, 1962b; Curtis and Watkins, 1963). Perhaps the strongest argument against a transmitter function for glutamic acid upon motoneu-

rones is that the equilibrium potential for the depolarization differs from that of the synaptically induced depolarization (Curtis, 1962b). The subcellular localization of glutamic acid also argues against a transmitter function (see section IV C).

The failure to demonstrate an effect of carbachol on the excitability of primary afferent fibres supports other pharmacological investigations (Eccles, Schmidt and Willis, 1963a; Kiraly and Phillis, 1961) which suggest that spinal presynaptic inhibition does not involve cholinergic mechanisms. Neither does adrenergic transmission seem to be involved since nor-adrenaline had no significant effect.

The action on terminals of two drugs, picrotoxin and strychnine, which block presynaptic and postsynaptic inhibition respectively (see Eccles et al., 1963a) was also investigated. The failure of picrotoxin to alter the excitability of primary afferent terminals suggests that the reduction in presynaptic inhibition is due not to a direct action on the excitability of the terminals, but that picrotoxin in some way prevents the transmitter from depolarizing the terminals.

Strychnine abolishes postsynaptic inhibition without affecting postsynaptic excitation (Bradley, Easton and Eccles, 1953; Fatt, 1954; Curtis, 1962, 1963). Thus the depression of the excitability of the primary afferent fibres observed in the present experiments was probably unrelated to its effect on postsynaptic inhibition. Consistent with this interpretation is the observation that relatively large electrophoretic currents were necessary to reduce excitability, whereas diffusion from the electrode is sufficient to abolish postsynaptic inhibition (Curtis, 1962). The depression of the excitability of

primary afferent fibres may be related to the local anaesthetic type of action which has been demonstrated on amphibian and mammalian nerve (Peugnet and Coppée, 1936; Bouman, 1937; Heinbecker and Bartley, 1939; Coppée and Coppée-Bolly, 1941; Erlanger, Blair and Schoepfle, 1941; Alving, 1961). However, the possibility cannot be excluded that strychnine has a predilection for inhibitory terminals, in the same way that it is more effective on some nerve fibres than it is on others (Peugnet and Coppée, 1936; Heinbecker and Bartley, 1939).

Thus the transmitter responsible for depolarizing afferent nerve terminals and so producing presynaptic inhibition remains elusive, as do the transmitters operating at most other synapses in the central nervous system. However, the present experiments have served to eliminate some of the possibilities.

F) General discussion

It is my intention in this section to summarize the main findings reported in the preceding sections, and to refer briefly to some recent data which might have a bearing on the nature of central synaptic transmitters.

With the demonstration that the cholinomimetic substance in synaptic terminals is ACh and not a related compound (section IV B), the status of ACh as a central transmitter now seems secure. It occurs naturally and is stored in the nerve-terminals in synaptic vesicles (criterion 1, see section IV C); it is synthesized by an enzyme in the presynaptic terminal (criterion 2, see section IV C); cholinesterase is present, probably in the postsynaptic membrane attached to the terminal (criterion 3, see section IV C); administration of ACh near nerve cells, the Renshaw cell in particular, mimics the action of the transmitter released by nervous stimulation (criterion 4, see section V); ACh is released from nervous tissue and the amount is increased when nervous pathways are activated (criterion 5, see Angelucci, 1956; Elliott, Swank and Henderson, 1950; MacIntosh and Oborin, 1953; Mitchell, 1961a,b, 1963; Mitchell and Phillis, 1962; Mitchell and Szerb, 1962; Szerb, 1963); pharmacological interactions on the Renshaw cell are similar to those of the naturally released transmitter (criterion 6, see section V) but this criterion has not been met at other cholinoceptive receptors in the central nervous system (see section V). It has not been shown conclusively that the action of ACh on neurones is localized to the subsynaptic membrane (criterion 4), but this criterion may not be obligatory (see section IVA).

A number of other substances of interest as possible transmitters occur in the C.N.S., but in section IV C it was concluded that only for substance P was there good evidence of storage in nerve terminals. So far there is no adequate evidence of a direct action of substance P on central neurones, and attempts to demonstrate its release from the C.N.S. have been unsuccessful (Gaddum, 1962, 1963). Gaddum (1963) has aptly summed up the position regarding the release of substances from central nervous tissue in the following words; 'There is no good evidence that any substance, except ACh, is liberated as a chemical transmitter in the central nervous system'. There is no need to say more.

The following comments on the action of drugs on central neurones are restricted to the results which have been obtained when the compounds are administered into the immediate surroundings of the neurones under observation, because the results obtained by systemic administration are difficult to evaluate (Curtis, Phillis and Watkins, 1961).

With the advent of the technique of micro-electrophoresis (see review by Curtis, 1964) a great deal has been learned about the pharmacology of central neurones, but with the single exception of the synapses on Renshaw cells, there are still great gaps in our knowledge of the substances which are involved in synaptic transmission at particular sites.

Many neurones in different parts of the C.N.S. are sensitive to ACh, but there is a dearth of evidence to support the contention that the cholinoceptive receptors are concerned in synaptic trans-

mission at these sites (section V). There is still less evidence that substances other than ACh are the transmitters at any particular site.

Rothballer (1959) obtained activation of the electro-encephalogram when micro-injections of catecholamines were made into the brain-stem reticular formation and Trzebski (1961) observed an increased activity of nearby neurones in the reticular formation after micro-injections of catecholamines. Bradley and Wolstencroft (1962) found that the electrophoretic application of nor-adrenaline caused excitation of some, and inhibition of other reticular neurones in decerebrate cats, a finding which confirmed the earlier observations of Bradley and Mollica (1958), who used systemic administration. However, Curtis and Koizumi (1961) failed to detect any effect with catecholamines applied electrophoretically to single neurones in the brain stem, a discrepancy which may be due to their use of anaesthetics. Although the evidence strongly suggests that these amines do have an action on reticular neurones, it by no means proves that they function as transmitters at these sites.

Recently, Bloom, Von Baumgarten, Oliver, Costa and Salmoiraghi (1964) showed that the adrenaline-antagonists, dibenamine and tolazoline, reduced the inhibition of olfactory mitral cells produced by stimulation of the lateral olfactory tracts, and also decreased the inhibition of spontaneous activity caused by the electrophoretic application of nor-adrenaline. Since no effect was obtained with atropine, dihydro-β-erythroidine, hexamethonium or physostigmine, even though acetylcholine produced an effect similar to that of nor-adrenaline, it was

suggested that an adrenergic mechanism was involved in lateral olfactory tract inhibition. The evidence for this is not convincing. Von Baumgarten, Bloom, Oliver and Salmoiraghi (1963) were unable to demonstrate that the acetylcholine-evoked inhibition was modified by physostigmine, atropine or dihydro-β-erythroidine; hence no particular significance can be attached to the failure of these substances to affect lateral olfactory tract inhibition. Furthermore it is well established that adrenergic blocking agents may also block the actions of acetylcholine (Fleckenstein, 1952; Boyd, Burnstock, Campbell, Jowett, Shea and Wood, 1963), and Bloom et al., (1964) did not test the effect of dibenamine or tolazoline on the response to acetylcholine.

Catecholamines have also been tested at other sites in the nervous system. The most extensive investigations are those of Curtis and Davis (1962) on neurones in the lateral geniculate body, and those of Krnjevic and Phillis (1963a,b and c) on neurones in the cerebral cortex. Crawford and Curtis (personal communication) have also obtained results similar to those of Krnjevic and Phillis (1963a, b, c) on cortical neurones. At both sites the electrophoretic application of catecholamines can depress spontaneous activity and the responses of the neurones to synaptic excitation; dopamine is more effective than either adrenaline or nor-adrenaline. However, on cortical cells, but not on geniculate neurones, the excitant effect of acidic amino acids is also blocked. The reduction of the effect of amino acids on the cortex, coupled with the probability that the extra-neuronal concentrations of the amines were high, led Krnjevic and Phillis (1963a) to suggest that

the effects on these cells were associated with non-specific actions. Such non-specific actions may also explain the actions of catecholamines at other sites (e.g. on hypothalamic neurones: Bloom, Oliver and Salmoiraghi, 1963).

Since the excitant effects of amino acids on geniculate neurones were not blocked by catecholamines in concentrations which blocked synaptic excitation, it was postulated (Curtis and Davis, 1963) that the catecholamines could be competing with the excitatory transmitter at postsynaptic receptor sites, and may therefore be structurally related to the transmitter. However, an action on presynaptic terminals could not be excluded.

Owman (1964) has made the interesting observation that nerve terminals in the pineal body, and elsewhere under special conditions, may take up amines from the extra-neuronal environment in vivo. If terminals in the geniculate body have the same ability to take up amines, then it is possible to explain the inability of catecholamines to block the postsynaptic excitatory effects of amino acids at concentrations which block synaptic transmission. The block of synaptic transmission may be envisaged as being due to an action on the terminals of a similar non-specific type as that observed on cortical neurones.

Curtis and Davis (1962, 1963) and Krnjevic and Phillis,(1963a) also investigated the actions of various indolic substances on geniculate and cortical neurones respectively. At both sites the action of indoles was qualitatively similar to that of catecholamines, but there were marked differences in relative potencies: on geniculate cells the hydroxytryptamines, in particular 4-hydroxytryptamine, were

more potent than the catecholamines, whereas on cortical neurones dopamine was one of the most potent compounds tested. Thus not only do the effects of indoles on geniculate neurones differ from the effects on cortical neurones in a qualitative fashion, but they also differ quantitatively. These quantitative differences may also be due to uptake by the terminals in the geniculate body, but such uptake has yet to be demonstrated. Thus it is the author's opinion that the experiments which have so far been carried out on the effects of catecholamines and of indoleamines on central neurones have given no firm support to the view that the transmitters operating at these sites are related to the substances which have been tested. Indeed, if the interpretation of the subcellular distribution studies is correct (see page 72), this lack of support is not surprising.

In addition to the depressant effects which we have been discussing, excitant actions have been noted on reticular neurones (Bradley and Wolstencroft, 1962), on cortical neurones (Krnjevic and Phillis, 1963a,b,c) and on olfactory neurones (Von Baumgarten, Bloom, Oliver and Salmoiraghi, 1963), but again there is no evidence to suggest that these excitant effects are in any way related to those of excitatory transmitters at these sites.

In previous sections (sections IV C and IVE) the role of amino acids in conventional transmission and in presynaptic inhibitory phenomena were discussed, and the conclusion was reached that they do not function as transmitters. In keeping with this conclusion, Gaddum (1962) was unable to obtain evidence for the release of substances with Factor I activity from the central nervous system.

Another observation merits comment in the present context: Ramwell and Shaw (1963) have shown that an unidentified oxytocic principle is releaed from the cortex when it is stimulated directly, transcallosally or through peripheral nervous pathways. Furthermore, this substance may be extracted from guinea pig or ox brain. It remains to be seen whether, like acetylcholine, the substance is localized in synaptic terminals, and whether it has pharmacological properties which would be appropriate for a synaptic transmitter.

Finally, it was shown in section IV D that it was possible to purify an acetylcholine-containing fraction sufficiently to eject the ACh from a micro-electrode in adequate amounts to excite single Renshaw neurones. Although the method had drawbacks, which perhaps explained the failure to detect other substances with actions on neurones, it is felt that the technique offers new opportunities for the detection and ultimate characterization of other transmitters in brain extracts.

V) THE PHARMACOLOGY OF CENTRAL CHOLINOCEPTIVE RECEPTORS

A) Introduction

In the preceding section, it has been emphasized that acetylcholine is the only substance for which there is adequate evidence of a transmitter function in the mammalian central nervous system. Furthermore, the most satisfactory evidence is that obtained from studies on Renshaw cells.

Renshaw cells are situated in the ventro-medial part of the spinal cord and are monosynaptically activated by antidromic volleys in the appropriate ventral roots (Renshaw, 1946; Eccles, Fatt and Koketsu, 1954; Eccles, Eccles and Fatt, 1956; Frank and Fuortes, 1956; Curtis and Eccles, 1958a,b; Longo, Martin and Unna, 1960; Eccles, Eccles, Iggo and Lundberg, 1961; Haase, 1963; Wilson and Talbot, 1963). This monosynaptic activation results in a characteristic high frequency discharge. There is now a great deal of evidence that transmission of the nerve impulse from the terminals of the motor axon collaterals onto these cells is effected by a cholinergic mechanism (Eccles et al., 1954; Eccles et al., 1956; Curtis and Eccles, 1958a,b). Renshaw cells are also activated by dorsal root volleys (Renshaw, 1946; Frank and Fuortes, 1956; Curtis, Phillis and Watkins, 1961), and this excitatory pathway is probably non-cholinergic (Curtis et al., 1961).

The earlier investigations (Eccles et al., 1956; Curtis and Eccles, 1958a) showed that the cells were sensitive to 'nicotinic'

substances (Dale, 1914) and relatively insensitive to 'muscarinic' substances. The response to orthodromic activation via the ventral root was blocked by dihydro-β-erythroidine (see also Longo, et al., 1960), but not by atropine. These results suggested that the acetylcholine receptors were of the 'nicotinic' type. This conclusion will be discussed in section (V B) and additional evidence will be produced which shows that there are both nicotinic and muscarinic receptors on the same neurone. In the discussion, these results will be compared with those obtained at different cholinoceptive sites by other investigators.

There is evidence that a depression follows the initial high frequency firing in response to a ventral root volley. A conditioning stimulus applied to a ventral root depressed the response to a second stimulus for periods up to 100 msec (Renshaw, 1946; Eccles et al., 1954). There was also a reduction in a test excitatory postsynaptic potential, recorded intracellularly, when this was preceded by a conditioning volley in the ventral root (Eccles et al., 1961). Furthermore, Frank and Fuortes (1956) and Curtis and Eccles (1958a) noted that the spontaneous discharge was reduced in the period following activation of the cell by ventral root stimulation. The latter investigators also observed that the frequency of firing of some cells declined during the electrophoretic administration of ACh with a constant ejecting current. Desensitization to ACh, but not to nicotine, was also observed by Eccles et al., (1956) after intra-arterial injection, although this was not necessarily due to a direct action on the Renshaw cell. During a more

intensive investigation of the action of cholinomimetic substances on Renshaw cells, it was noted that desensitization was regularly observed when acetylcholine was administered electrophoretically for a sufficiently long period. A more detailed study of the desensitization caused by cholinomimetic agents, and by activation of the Renshaw cell after stimulation of the ventral roots, was therefore carried out. An attempt was made to distinguish between specific desensitization, which only affects the action of substances reacting with the same receptor, and non-specific desensitization, which affects substances reacting with different receptors.

Paton (1961) has proposed a 'rate theory' which introduces the concept that the magnitude of the response produced by a substance is determined by the rate at which it combines with receptors. Inherent in this theory is the conclusion that, when a substance has combined with the receptor, the number of receptors available for occupation decreases, and so the rate of combination between drug and receptor also decreases and leads to a reduction in the response. Hence, the pattern of the response to a particular drug depends to a great extent on the rate of dissociation of the drug-receptor complex; potent excitants tend to have fast rates of dissociation from the receptor molecules, whereas specific blocking agents have slow rates of dissociation. The theory also predicts that, in the continued presence of an excitant, the effect will decrease to an equilibrium and the rate and extent of such a 'fade' will depend upon the concentration of the drug and its association and dissociation constants with regard to the receptor molecules.

This 'fade' phenomenon and the action of specific blocking agents are produced by the same mechanism, which is termed specific desensitization. In contrast, non-specific desensitization affects the reaction of the tissue to drugs acting on a variety of receptors. It may be due to some consequence of excitation of the cell, such as exhaustion of some intra-cellular component (Paton, 1961). However, it is possible that a non-specific desensitization could also be produced by blocking a process intermediate between occupation of the receptor by the agonist molecule and excitation; this intermediate stage would have to be the same for excitation initiated by the interaction of agonists with different receptors (see Discussion).

The excitatory action of acidic amino acids upon Renshaw neurones is unaffected by the administration of dihydro-β-erythroidine, which blocks the action of cholinomimetic substances (Curtis, Phillis and Watkins, 1960, 1961). Since the actions of amino acids and cholinomimetics seem to involve different receptors, it was possible to differentiate between specific and non-specific desensitization by employing cholinomimetic substances and an excitant amino acid (DL-homocysteic acid) in these studies.

B) On the presence of nicotinic and muscarinic receptors on the same Renshaw neurone

i) Results

a) The relative potencies of cholinomimetic substances

The relative potencies of a wide range of cholinomimetic substances were determined on Renshaw cells, and on a variety of other preparations in which the effects are typically nicotinic or muscarinic. The potencies on Renshaw cells were determined by comparing the electrophoretic currents required to produce equal effects when the compounds were ejected from the micro-pipettes. In such comparisons it was necessary to have the substances in equimolar concentrations, since diffusion from the micro-pipette was related to the concentration of the cholinomimetic within it. Other factors also control the rate at which substances are ejected electrophoretically from micro-pipettes (Curtis, 1964). Therefore, the relative potencies so determined are only approximate.

Inspection of Table 5 shows that the relative potencies of various cholinomimetic substances on Renshaw cells were similar to those obtained when the tests were carried out on peripheral nicotinic receptors. Furthermore, the effects on Renshaw cells bore a closer resemblance to those on ganglionic receptors than on the nicotinic receptors of skeletal muscle. In Fig. 28, the substances are arranged in descending order of the potency determined on the blood pressure of the atropinized cat (indicated by filled circles). The approximate potencies on Renshaw neurones are indicated by the open circles; where the potencies were more than four times as great as that of ACh, or

less than one fifth of the potency of acetylcholine, this is indicated by arrows pointing up or down respectively. The figure demonstrates that there was a reasonable correlation between the effectiveness on Renshaw neurones and on the blood pressure of the atropinized cat, but there was no similarity to the effect on the muscarinic receptors of the guinea pig ileum (crosses).

Although muscarine and acetyl-β-methylcholine were very ineffective when tested on peripheral nicotinic receptors (see Table 5), they excited Renshaw neurones (Figs. 29, 30, 32, 33, 49), but the potencies were less than that of acetylcholine. The effects of muscarine and acetyl-β-methylcholine were variable. In some experiments (see Fig. 32), there was a gradual increase in the rate of firing during the application and a slow recovery when the ejecting current was terminated. In most experiments (see Figs. 29, 33, 49), there was either no firing or a slow onset of firing during the application but the rate of firing increased when the electrophoretic current was terminated and then slowly declined to the resting level. When this type of effect occurred, the rate of firing attained during the application tended to remain constant despite an increase in the ejecting current (Fig. 30), but the rate attained after the current was terminated rose progressively. In other experiments (see Fig. 49), firing did not commence until the ejection was terminated. The excitatory actions of dl-muscarine and acetyl-β-methylcholine indicated that there might be muscarinic receptors in addition to nicotinic receptors on single Renshaw neurones. Further investigations were therefore carried out to test this postulate.

b) The action of acetylcholine-antagonists on the excitatory effects of cholinomimetic substances

As found by previous investigators (Eccles, Fatt and Koketsu, 1954; Curtis and Eccles, 1958a; Curtis, Phillis and Watkins, 1961) the effect of acetylcholine could be reduced or abolished by the administration of dihydro-β-erythroidine. Fig. 31 A (right hand side) shows that the passive diffusion of dihydro-β-erythroidine from the electrode caused a marked reduction in the response to acetylcholine. When the antagonist was ejected by electrophoretic currents as high as 20 nA, which completely suppressed the response to acetylcholine, the response to an excitant amino acid, DL-homocysteic acid, was unaffected, or possibly slightly increased.

Dihydro-β-erythroidine was about 200 times less effective than atropine in blocking the muscarinic action of acetylcholine upon the guinea pig ileum, but was more than 200 times as effective in blocking the nicotinic effect of acetylcholine on the toad rectus (Ryall, unpublished). Since the excitant effect of acetylcholine on Renshaw cells was abolished by low concentrations of dihydro-β-erythroidine, the action of acetylcholine appeared to be due largely to an interaction with nicotinic receptors. It was therefore of interest to compare the effects of dihydro-β-erythroidine and atropine on the responses of the Renshaw cells to acetylcholine and the cholinomimetic, acetyl-β-methylcholine, which may have been producing its effect by an interaction with muscarinic receptors.

Attempts to demonstrate a differential effect of electrophoretically administered atropine yielded only inconclusive results;

this method of application frequently depressed the responses to both cholinomimetics and to excitant amino acids. This non-specific action of atropine has been previously reported and was attributed to a local anaesthetic type of effect on the cell membrane(Curtis and Phillis, 1960).

A clear demonstration of the presence of both nicotinic and muscarinic receptors was obtained when the atropine was injected intravenously in small doses. Fig. 32 illustrates the results obtained in such an experiment. In Fig. 32 A, control responses to acetylcholine and acetyl-β-methylcholine were obtained. Dihydro-β-erythroidine was then ejected from the micro-pipette for a period of 3 min before the records in Fig. 32 B were obtained. At this time (Fig. 32 B) the response to acetylcholine was reduced by about 60 per cent, but the effect of acetyl-β-methylcholine was only slightly reduced during the early part of the response. The electrophoretic ejection of dihydro-β-erythroidine was then terminated. (The specific action of dihydro-β-erythroidine is also illustrated in Fig. 38). Four minutes later, when the response to acetylcholine had recovered (as in Fig. 32 C), 0.1 mg/kg of atropine sulphate was injected intravenously, and six minutes later the records shown in Fig. 32 C were obtained; the effect of acetyl-β-methylcholine was greatly reduced, whereas the response to acetylcholine was unchanged. Much higher doses of atropine, up to 1.7 mg/kg (Fig. 32 D and E), caused only a slight reduction in the response to acetylcholine; the excitant effect of dl-muscarine was also blocked by atropine. Thus, the effect of acetylcholine appeared to be due mainly

to an interaction with nicotinic receptors and that of acetyl-β-methylcholine to an interaction with muscarinic receptors, although the latter substance may have some nicotinic action.

Since acetylcholine reacts with peripheral nicotinic and muscarinic receptors, it was of interest to see whether an effect on muscarinic receptors could be demonstrated after the nicotinic receptors had been blocked by dihydro-β-erythroidine. Dihydro-β-erythroidine usually completely suppressed the firing induced by acetylcholine. However, when the amount of acetylcholine ejected was increased, after blockade of the nicotinic receptors with dihydro-β-erythroidine, acetylcholine caused the cell to fire (Fig. 33 B). This excitation was much slower in time course than the response obtained initially, but was similar to the action of acetyl-β-methylcholine on the same cell and was reduced by the administration of atropine (Fig. 33 C).

Another piece of evidence, suggesting a dual action of acetylcholine, was obtained in a few experiments when large amounts of acetylcholine were ejected (Fig. 34). In these experiments the recovery appeared to consist of a fast phase and a slow phase. This is clearly seen in Fig. 34 after acetylcholine was ejected with a brief current pulse of 150 nA, but was less evident with lower concentrations. Fast and slow phases in the recovery have also been observed following the administration of carbamylcholine. However, the time course of the slow phase following the ejection of carbamylcholine was extremely prolonged, and in some experiments, particularly after the ejection of large amounts, it exceeded 1 hr in duration. In one experiment when atropine (0.1 mg/kg) was injected intravenously during this slow

phase of the recovery after carbamylcholine, the rate of firing decreased to the original level.

These experiments have provided the first conclusive evidence that both nicotinic and muscarinic receptors may occur on the same neurone, but they yield no information regarding the spatial relationships of the two types of receptor upon the membrane of the Renshaw cell.

The actions of several other acetylcholine-antagonists were also investigated. Hexamethonium,d-tubocurarine and tetraethylammonium, applied electrophoretically to Renshaw neurones, blocked the action of acetylcholine but not that of acetyl-β-methylcholine nor that of DL-homocysteic acid.

Tetraethylammonium (TEA) had a very brief depressant action on the firing induced by acetylcholine (Fig. 35 A) and on the firing induced by nicotine (Fig. 35 B and C). When TEA was administered electrophoretically at the same time as nicotine (Fig. 35B), the neurone did not fire but the cell began firing when the ejection of both nicotine and TEA was terminated. The firing frequency climbed to a maximum, which exceeded the resting rate of discharge, and then declined with a time course similar to that observed following a control test with nicotine alone. When TEA was ejected up to 3 min after the ejection of a larger amount of nicotine (Fig. 35 C), a brief reduction in the firing frequency was observed.

This experiment was of interest since it demonstrated that the rate of removal of nicotine from the site of application was a slow process, lasting for several minutes; if the prolonged action of nicotine was due to prolonged receptor occupation and nicotine was

rapidly removed from the site of application, then it is impossible to explain why, after receptor occupation had presumably been prevented by the simultaneous application of TEA (Fig. 35 B), the rate of firing rose when the ejections of nicotine and TEA were terminated, and then declined with a time course similar to that of a control ejection of nicotine alone. However, if the prolonged action of nicotine was due to slow removal, then the results obtained in Fig. 35 B and C are easily explained on either receptor occupation theory (see reviews by Ariens and Simonis, 1964a, b) or rate theory (Paton, 1961).

The rates of offsets of the excitant effects of other cholinomimetic substances (see Fig. 29) may therefore be determined largely by the rates at which they are removed, rather than by the relative stabilities of the drug-receptor complexes.

The rates of removal are probably determined by the rates of inactivation by cholinesterase, by diffusion and possibly by uptake by adjacent tissues. It is also likely that these factors limit the site of action of different electrophoretically applied drugs to different areas of cell membrane. This conclusion is important when interpreting the differential effect of dihydro-β-erythroidine on the orthodromic responses of Renshaw cells and on the action of cholinomimetic substances (section V B c).

c) <u>The effect of acetylcholine-antagonists on orthodromic responses</u>

Since good evidence for the presence of both nicotinic and muscarinic receptors was obtained by the pharmacological tests which have just been described, it was of interest to determine whether both types of receptors were involved when the cell was synaptically activated.

Following a single stimulus to the ventral root, a Renshaw cell fires initially at a high frequency which then declines over a period ranging from 20-50 msec (Fig. 36; see also Renshaw, 1946; Eccles et al., 1954, 1956; Longo et al., 1960). This initial response is followed by a period of about 200 msec, during which spikes are not observed (see Figs. 36 and 37 A). This period, which will be called the pause, was also noted by Frank and Fuortes (1956) and by Curtis and Eccles (1958a). After the pause, the firing rate of the cell increases to a maximum at about 1 sec after the stimulus and then gradually returns to the initial background rate of firing within 3 sec (Fig. 37 A). The maximum frequency of firing during this late response, which was previously observed by Frank and Fuortes (1956), was always lower than the frequency attained during the initial response (see Fig. 37 A). An initial response, smaller than that following ventral root stimulation, and a pause, but not a late response, were also observed when Renshaw cells were fired by volleys in the dorsal roots (Fig. 37 B and C).

It is well known that the initial response following activation of ventral root fibres, but not the initial response following stimulation of dorsal root fibres, is blocked by dihydro-β-erythroidine (see Eccles et al., 1956; Curtis and Eccles, 1958b; Curtis et al., 1961). Presumably, muscarinic receptors are not involved in the former response. Fig. 38 shows the effect of dihydro-β-erythroidine on the number of spikes in the initial response following ventral root stimulation (Fig. 38 A), on responses to electrophoretically

administered acetyl-β-methylcholine or nicotine (Fig. 38 B), and on the responses to acetylcholine or n-butyrylcholine (Fig. 38 C). This experiment took over 2 hr to complete. During this period, the effect of an excitant amino acid, DL-homocysteic acid, which is unaffected by dihydro-β-erythroidine (see Fig. 31), tended to vary, due to minor changes in the relative positions of the electrode and the cell under observation. In order to allow for this variation, the responses to the cholinomimetics are expressed as percentages of the control responses to DL-homocysteic acid.

Dihydro-β-erythroidine caused a marked reduction in the orthodromic response and in the responses to nicotine, acetylcholine and n-butyrylcholine, but there was no depression of the maximum firing frequency attained by the electrophoretic ejection of acetyl-β-methylcholine. It is significant that all three substances whose effects were blocked by dihydro-β-erythroidine possessed marked nicotine-like effects (Table 5). However, following the cessation of the current ejecting dihydro-β-erythroidine, the recovery to orthodromic activation and nicotine followed a similar, prolonged time course, but the recovery to acetylcholine and n-butyrylcholine was rapid. The transmitter substance liberated during synaptic activity of the terminals of the motor axon collaterals is assumed to be acetylcholine, but the different time courses of the recovery to acetylcholine and orthodromic activation appear to be at variance with this hypothesis. This discrepancy may be due to the fact that the cholinomimetics, acetylcholine and n-butyrylcholine, are limited in their sites of action to

a small proportion of the total area of the receptor surface (see page 117). However, nicotine may affect more of the available receptor surface, in the same way that orthodromic activation presumably leads to the release of acetylcholine over a large proportion of the cell membrane. In order to produce a given response, by activation of receptors on a small part of the available receptor surface, the concentration of cholinomimetics, such as acetylcholine or n-butyrylcholine, would be larger than that required to produce the same response with nicotine (or with the transmitter liberated by ventral root stimulation), which produce their effects on a larger area of receptor surface. A given concentration of dihydro-β-erythroidine would therefore block the action of nicotine and the synaptic transmitter more effectively than that of acetylcholine or n-butyrylcholine. An alternative explanation is that the receptors with which the transmitter and nicotine react are different from those with which acetylcholine and n-butyrylcholine react. This is unlikely because both types of receptors appear to be blocked by dihydro-β-erythroidine.

Dorsal root volleys fire Renshaw neurones, but there was no late response (see Fig. 37). The initial response is unaffected by dihydro-β-erythroidine, and therefore the mechanism was said to be non-cholinergic (Curtis, Phillis and Watkins, 1961). Renshaw neurones have now been shown to have muscarinic receptors as well as nicotinic receptors, and it was possible that transmission from the terminals of the interneurones activated by dorsal root volleys was cholinergic, but that it involved muscarinic receptors. However, the initial response recorded from Renshaw cells following dorsal root stimulation

was unaffected by atropine, administered intravenously in doses up to 1 mg/kg. Thus, the conclusion that this transmission is non-cholinergic appears to be correct.

Another possibility is that muscarinic receptors are involved in the production of the late response following ventral root stimulation. The effect of atropine on this late response is shown in Figs. 39 and 40 B. When atropine was administered intravenously in a dose of 0.1 mg/kg, the late response was completely abolished, but there was no effect on the initial response. It is of interest to note that the pause seemed to be prolonged, presumably because the late response normally tends to overcome the depression of firing during the pause, which is thought to be due to desensitization (see section V D). Even a larger dose of atropine (1 mg/kg) had no effect on the initial response (Fig. 39), but the rate at which the cell was firing spontaneously, which had already been slightly reduced by the preceding dose of atropine, was reduced still further. Thus, much of the background activity of Renshaw cells appears to be due to the interaction of acetylcholine with muscarinic receptors.

The effect of dihydro-β-erythroidine (Fig. 40 A) demonstrates the clear distinction between the initial response, which involves interaction with nicotinic receptors, and the late response, which involves interaction with muscarinic receptors. In this experiment the dihydro-β-erythroidine (20 mM in 165 mM NaCl) was ejected from the micro-pipette with an electrophoretic current of 25 nA. This reduced the average rate of firing during the initial response from 700/sec

to about 150/sec. In contrast, the maximum firing rate during the late response increased from 50/sec to 70/sec. This increase may be partly due to a direct stimulant effect of dihydro-β-erythroidine; when the electrophoretic ejection was continued, the background firing of the cell tended to increase, expecially when large electrophoretic currents were used to eject the antagonist (see also, Curtis, et al., 1961). This direct excitant effect of dihydro-β-erythroidine may be the consequence of interaction with nicotinic or even muscarinic receptors.

The late response following a single ventral root stimulus was typically prolonged and lasted for periods of up to 4 sec (see Fig. 37A, 39 and 40). Since the late response and the spontaneous activity were both reduced by atropine and therefore presumably involved the interaction of acetylcholine with muscarinic receptors, it seemed possible that the spontaneous activity was the result of the summation of the small late responses arising from random synaptic bombardment of the cells. It was therefore of interest to determine whether the late responses summed on repetitive stimulation of the ventral roots.

Some of the results obtained in one such experiment are shown in Fig. 41. In this figure the initial responses have been omitted. The filled circles show the late response following a single stimulus and the dotted line represents the rate of discharge under resting conditions. When the ventral root was stimulated at a rate of 3/sec for 3 sec, the late response following the last stimulus in the train had the form indicated by the open circles. The response rose to a

maximum more slowly than it did after a single stimulus, the maximum frequency was higher and the duration of the discharge was more prolonged; there was no noticeable effect when the root was stimulated at 1/sec or less. At higher frequencies of stimulation, there was an even slower rise to the maximum firing frequency following the last stimulus in the train and the duration of the response became progressively more prolonged; at a frequency of 200/sec, the late response lasted about 50 sec. The progressively slower rise of the late response with increasing frequency of stimulation may be correlated with a progressively more prolonged desensitization during the initial response, as indicated by the fact that the number of spikes elicited during the initial response fell as the frequency of stimulation increased.

ii) Discussion

The demonstration that Renshaw cells have both nicotinic and muscarinic receptors provides the first conclusive evidence that both types of receptor may be present on a single neurone. The pharmacology of the Renshaw cell is in fact very similar to that of sympathetic ganglia, where both nicotinic and muscarinic receptors have also been demonstrated (Ambache, Perry and Robertson, 1956; Eccles and Libet, 1961; Jones, 1963; Takeshige, Pappano, Groat and Volle, 1963; Takeshige and Volle, 1963; Libet, unpublished). However, there is as yet no clear evidence that in ganglia both types of receptor are present on the same neurone.

Atropine is ineffective on the initial response recorded from Renshaw cells following volleys in the ventral roots. However, following ventral root stimulation, there is a late response, which is

completely suppressed by atropine; this late response is presumably similar to the late negative wave which has been recorded from sympathetic ganglia following preganglionic stimulation and which is also suppressed by atropine (Eccles and Libet, 1961; Libet, unpublished). Since atropine did not reduce the initial response, it was possible that the muscarinic receptors involved were located beneath synaptic terminals different from these which belong to the motor axon collaterals. Renshaw cells are known to be activated by volleys in dorsal root fibres (Renshaw, 1946; Frank and Fuortes, 1956; Curtis et al., 1961), and transmission via this pathway is not blocked by dihydro-β-erythroidine (Curtis et al., 1961). However, atropine did not block the effects of dorsal root stimulation. Nevertheless, it is possible that other excitatory pathways onto Renshaw cells may have a final cholinergic step which involves muscarinic receptors. Alternatively, the muscarinic receptors may be extra-synaptic, as suggested for the muscarinic receptors in sympathetic ganglia (Takashige and Volle, 1963).

Muscarinic receptors were involved both in the late response following maximal ventral root stimulation and in spontaneous activity. Furthermore, the late response was greatly prolonged by repetitive stimulation of the ventral root, even at quite low frequencies. Therefore, the spontaneous activity of Renshaw cells may be caused by the slow release of acetylcholine from the terminals of motor axon collaterals and the subsequent combination of acetylcholine with the muscarinic receptors. However, the late response may not be significant under physiological conditions of synaptic activation, and it is

possible that acetylcholine derived from non-neuronal sources normally combines with these receptors. These muscarinic receptors may maintain a slight background of inhibition onto motoneurones that is sufficient to reduce the effects of random excitatory bombardment.

Many other neurones in the central nervous system are cholinoceptive, but there is no conclusive evidence for cholinergic transmission at any of these sites.

Krnjevic and Phillis (1963b, c) and Crawford and Curtis (unpublished) have shown that the effects of acetylcholine on Betz cells are reduced by atropine but not by dihydro-β-erythroidine and that muscarine and acetyl-β-methylcholine are potent excitants. Therefore, the receptors are presumably of the muscarinic type. Although intravenous doses of atropine tended to reduce the late, presumably polysynaptic, response which followed stimulation of the internal capsule or the thalamus (Krnjevic and Phillis, 1963b), this effect of atropine cannot be considered as conclusive evidence for cholinergic transmission involving muscarinic receptors on Betz cells. Since locally applied atropine briefly depressed synaptic activation and the response to glutamic acid, but caused a prolonged depression of the response to acetylcholine (Krnjevic and Phillis, 1963b), it seems likely that the only effect of atropine on the synaptic activation of Betz cells is a non-specific, local anaesthetic type of action, similar to that observed on other types of central neurones (Curtis and Phillis, 1960). Thus, the site of action of atropine on the late synaptic response recorded from Betz cells may be on the interneurones on the polysynaptic pathway. Furthermore, the possibility cannot be

excluded that these interneurones are subject to the excitatory effects of acetylcholine derived from extra-neuronal sources, and that, by removing this background of excitation, atropine may depress transmission at these synapses. In this context, it should be remembered that at other sites acetylcholine may also be derived from non-neuronal sources, for example, in ganglia (McInstry, Koenig, Koelle and Koelle, 1963) and in skeletal muscle (McIntyre, Downing, Bennett and Dunn, 1950; Straughan, 1960; Hayes and Riker, 1963; Mitchell and Silver, 1963).

Neurones in the cerebellum (Crawford, Curtis, Voorhoeve and Wilson, 1963b; Crawford and Curtis, 1964; McCance and Phillis, 1964), are also sensitive to acetylcholine. Here again the receptors involved appear to be mainly muscarinic in type, and there is no evidence that acetylcholine antagonists, such as dihydro-β-erythroidine or atropine, are able to block various synaptic inputs (Crawford and Curtis, 1964). However, it is possible that the cholinergic pathway has not yet been located.

Cholinoceptive neurones have been located in the medulla (Salmoiraghi and Steiner, 1963; Bradley, Dhawan and Wolstencroft, 1964), inferior colliculus (Curtis and Koizumi, 1961), reticular formation (Bradley and Wolstencroft, 1962), thalamus (Curtis and Andersen, 1962; Andersen and Curtis, 1964a, b), hypothalamus (Bloom, Oliver and Salmoiraghi, 1963), lateral geniculate body (Curtis and Davis, 1963) olfactory bulb (Von Baumgarten et al., 1963) and caudate nucleus (Bloom, Costa, Oliver and Salmoiraghi, 1964). At some of these sites the receptors appear to be nicotinic, or a mixture of both nicotinic

and muscarinic receptors (see Andersen and Curtis, 1964b) and at others there is insufficient information on the type of receptor involved. At all these sites, in common with Betz cells in the cerebral cortex, and Purkinje cells in the cerebellum, conclusive evidence of cholinergic transmission has yet to be produced.

Thus, the Renshaw cell remains unique in being the only neurone known to possess both nicotinic and muscarinic receptors, and for which there is adequate evidence for cholinergic transmission.

C) A hypothesis concerning muscarinic receptors

In the preceding section it was suggested that the muscarinic receptors on Renshaw cells could be extra-synaptic. Although no conclusive evidence in support of this suggestion was obtained on Renshaw cells, the anomalous results obtained when atropine is tested on the effects of cholinergic nerve stimulation of a number of organs (see review by Ambache, 1956) could be explained in terms of the following hypothesis. The hypothesis states simply that muscarinic receptors are extra-synaptic. A corollary to this is that where there are subsynaptic cholinoceptive receptors, these are nicotinic, but this does not imply that all nicotinic receptors are subsynaptic; it is well known that nicotinic receptors may be present in situations where there are no synapses, e.g. on sensory nerve-terminals (see Gray, 1959), and on mammalian C-fibres (see Ritchie and Armett, 1963), although the receptors at these locations may have no physiologically significant function.

It is well known that atropine blocks the effects produced by stimulation of many parasympathetic nerves but that some effects are resistant (Dale and Gaddum, 1930; Ambache, 1956). Dale and Gaddum (1930) speculated that the atropine-resitance was due to the liberation of acetylcholine in close proximity to receptors which were inaccessible to atropine. In contrast, when nerve stimulation was blocked by atropine, such intimacy was supposed not to exist. It will be seen that the present hypothesis is compatible with this view, but it is taken one step further. The atropine-resistant effects are considered to be mediated by acetylcholine across synapses (in the sense that has

been defined by De Robertis, 1958, 1959, and Eccles, 1964; see page 19), where the subjunctional receptors are nicotinic. The atropine-sensitive effects are considered to be those which involve the interaction of acetylcholine with extra-junctional, muscarinic receptors. Thus, it is of interest to note that recent electron-microscopic studies of the innervation of smooth muscle (Richardson, 1958, 1962; Merrillees, Burnstock and Holman, 1963) and of cardiac muscle (Fawcett and Selby, 1958; Trautwein and Uchizono, 1963) have failed to demonstrate conclusively the presence of synapses similar to those found on skeletal muscle, in ganglia and in the central nervous system; these investigators occasionally found structures in which the nervous elements were separated from muscle fibres by a distance of only a few hundred Angstrom units, but there was no differentiation of either structure similar to that found in synapses elsewhere. Thus, it is highly probable that the typical muscarinic receptors on smooth muscle and on cardiac muscle are extra-synaptic. Further studies are required to show whether there is any correlation between atropine-resistant cholinergic nerve stimulation and the occurrence of synapses in smooth muscles.

If the hypothesis is valid, then it is necessary to show that at those sites at which atropine-resitance is encountered there are nicotinic receptors on the smooth muscle fibres. Such a demonstration is hampered by the fact that the anatomical arrangement of smooth muscle is complex, and that ganglion cells, with associated nicotinic receptors, may be in close proximity to the smooth muscle fibres. However, there are a number of observations which are relevant. Gyermek (1961)

showed that the effects of muscarine and acetyl-β-methylcholine on the urinary bladder of the cat and dog were blocked by atropine but there was only a moderate reduction of the response to acetylcholine and no effect on the response to nicotine or nerve stimulation. Henderson and Roepke (1934, 1935) also carried out experiments on the urinary bladder. They found that the response to nerve stimulation was biphasic, consisting of a rapid contraction which was not maintained, followed by a slow maintained contraction. The slow responses were blocked by atropine and the rapid contractions were blocked by nicotine. Edge (1955) showed in the cat that, after the administration of atropine, both nerve stimulation and acetylcholine caused only rapid contractions of the bladder which were abolished by the administration of hexamethonium. Henderson and Roepke (1934, 1935) and Gyermek (1961) concluded that there were nicotinic receptors on the smooth muscle of the bladder. These results could all be easily explained if the rapid contractions were due to the interaction of released acetylcholine with subsynaptic nicotinic receptors on the smooth muscle, and the slow contraction was due to an interaction with extra-junctional muscarinic receptors. It then becomes unnecessary to postulate that the postganglionic innervation of the bladder is non-cholinergic (Henderson and Roepke, 1934, 1935).

Munroe (1953) showed that stimulation of the peri-arterial nerves produced a quick contraction of the intestine followed by a slow contraction, and that the quick response was more affected by ganglion-blocking drugs than was the slow contraction. Nicotine produces a contraction of the cooled intestine, in which nerve conduction is

blocked (Ambache, 1946). Thus, nicotine may have a direct action on intestinal muscle. In addition, Luco and Altamirano (1943) found that curare reduced the effects of stimulation of some postganglionic parasympathetic nerves, which again suggests that there may be some circumstances in which postganglionic neuro-effector transmission may involve the interaction of the released transmitter with nicotinic receptors.

The results obtained with a number of tissues therefore suggest that cholinergic nerve stimulation may produce two effects, a rapid effect which is insensitive to atropine and a slow effect which is sensitive. In these respects there is a close similarity to the results obtained in sympathetic ganglia (Eccles and Libet, 1961; Takeshige, Pappano, Groat and Volle, 1963; Takeshige and Volle, 1963) and on Renshaw cells (section V B). In accordance with the hypothesis, all fast responses may be considered to be the consequence of the interaction of released acetylcholine with subsynaptic nicotinic receptors and all slow responses may be due to an interaction with extra-synaptic muscarinic receptors, after diffusion from the site of liberation.

Skeletal muscle is unusual, in that the area of muscle fibre which is innervated is confined to a small region, the end-plate. The acetylcholine receptors seem to be predominently nicotinic but nevertheless, there is some evidence for the presence of muscarinic receptors. Raventos (1937) and Brown (1937) found that a diphasic response of the frog gastrocnemius muscle followed stimulation of its motor nerve. The initial rapid response was differentially sensitive

to curare and the second slower response was differentially sensitive to atropine. Abdon (1940) and Tum-Suden (1958) have shown, in a variety of species, that atropine blocks the effects of artificially administered acetylcholine more readily than it blocks neuro-muscular transmission; the analogy with smooth muscle is obvious.

If the muscarinic receptors are extra-synaptic, in accordance with the hypothesis, then experiments on skeletal muscle could provide direct evidence in support of the postulate.

When acetylcholine is applied by micro-electrophoretic techniques to single innervated muscle fibres, a depolarization is recorded only when the micro-pipette is positioned close to the end-plate region (see reviews by Thesleff, 1960 and Miledi, 1962). However, when the muscle is chronically denervated, acetylcholine sensitivity increases along the length of the fibre (Miledi, 1960a, b, 1962; Axelsson and Thesleff, 1959; Thesleff, 1960), although the end-plate region probably remains the most sensitive (Miledi, 1962); this increase in sensitivity has been attributed to the growth of extra-junctional receptors (Miledi, 1960a, b, 1962; Axelsson and Thesleff, 1959; Thesleff, 1960).

It is probable that many of these extra-junctional receptors are nicotinic since, in denervated muscle, the depolarization produced by the electrophoretic application of acetylcholine or of carbamyl-choline, or the contractions produced by the diffuse application of acetylcholine are reduced by d-tubocurarine (Axelsson and Thesleff, 1959; Elmquist and Thesleff, 1960). However, these experiments do not show conclusively that muscarinic receptors are absent from the extra-junctional regions of denervated skeletal muscle fibres; the excitant

action of acetylcholine on Renshaw cells is abolished by dihydro-β-erythroidine, but nevertheless muscarinic receptors are present (section V B).

There is some suggestive evidence that the receptors at the end-plate region differ from those which appear in denervated muscle. Although denervated muscle is slightly more sensitive than normal muscle to d-tubocurarine, the increase in sensitivity to acetylcholine and carbamylcholine is far greater (Jenkins, 1960). This is not the result to be expected if the additional receptors were all identical to those present in normal muscle since it implies that at least some of the new receptors are relatively resistant to d-tubocurarine. Another result which is difficult to explain if all the receptors on denervated muscle fibres are identical is that, in mammalian muscles, receptor desensitization occurs more slowly than it does in innervated muscle (Axelsson and Thesleff, 1959; Thesleff, 1960); different results were obtained in frog muscle by Miledi (1960a), but this may represent a species difference since the time course and extent of denervation-supersensitivity differs in frogs and mammals.

In experiments on denervated rat diaphragms (Ryall, unpublished experiments), it was found that acetylcholine produced a contracture which faded rapidly, despite the constant presence of the substance in the muscle bath, whereas acetyl-β-methylcholine caused the muscle to contract and the contraction was well maintained for long periods of time. It is therefore possible that the acetylcholine reacted with receptors which were rapidly desensitized, whereas acetyl-β-methylcholine reacted with receptors which were desensitized less readily. The

effect of acetyl-β-methylcholine was reduced by atropine in concentrations of about 20 μg/ml and was also partially reduced by d-tubocurarine. In addition, Dale and Gaddum (1930) showed that the contracture of the denervated diaphragm of kittens to acetylcholine was reduced by atropine.

While the data are by no means conclusive, they do indicate that muscarinic receptors may be present on denervated skeletal muscle and that these receptors may be located away from the end-plate region of the fibres. Experiments in which acetyl-β-methylcholine or muscarine are administered to localized regions of the membrane, in conjunction with studies on the effects of atropine and d-tubocurarine, should yield more conclusive data.

There appears to be a great deal of largely circumstantial evidence to support the hypothesis that muscarinic receptors are extra-synaptic and that subsynaptic cholinoceptive receptors are nicotinic. Furthermore, all known nicotinic effects seem to be concerned with rapid responses, whereas muscarinic actions produce relatively prolonged effects. In the nervous system, the prolonged muscarinic effects which are produced by the synchronous synaptic bombardment of sympathetic ganglion cells or of Renshaw cells may be functionally insignificant when the neurones are subjected to asynchronous bombardment under normal physiological conditions. Furthermore, it is difficult to exclude the possibility that the muscarinic receptors are normally involved, not only in the synaptic transmitter effects, but also in the mediation of local hormonal actions of acetylcholine, which could be derived from extra-neuronal sources; such local hormonal effects

may also occur in other tissues e.g. in the intestine (Feldberg and Lin, 1950) or in tracheal muscle (Carlyle, 1964) and may set the level of background activity.

Although the hypothesis may explain various anomalous results, such as the failure of atropine to block the effects of parasympathetic nerve stimulation or the inability to locate the cholinergic pathways onto central neurones which have muscarinic receptors, final proof of the postulate awaits conclusive experimental data.

D) Desensitization phenomena and the limitation of synaptic excitation of Renshaw cells

i) Results

a) Desensitization produced by cholinomimetic substances

Curtis and Eccles (1958a) showed that, when acetylcholine was administered electrophoretically to Renshaw cells, the firing rate increased rapidly to a maximum and, in some cells, despite the maintenance of a constant ejecting current, it decreased. Further experience has shown that this decline in the frequency of firing is regularly observed with acetylcholine and other cholinomimetics, provided that the ejection is continued for a sufficiently long period. In some instances, this decline in the response appeared to be associated with specific desensitization and in others the desensitization appeared to be predominently non-specific in nature.

Paton's theory (Paton, 1961) predicts that all excitants should show some degree of specific desensitization (see Introduction). Specific desensitization of Renshaw cells to test doses of acetylcholine could be demonstrated when the frequency of firing produced by the prolonged administration of acetylcholine remained comparatively low. Results from one such experiment are shown in Fig. 42; two barrels (1 and 2) of the five barrelled pipette contained 0.5 M acetylcholine bromide and a third barrel contained 0.2 M DL-homocysteic acid (sodium salt; pH 8). It was thus possible to test the acetylcholine sensitivity of this cell without the complications which might have arisen if both the test and the conditioning doses of ACh had been

ejected from the one micro-pipette. Throughout this series, the Renshaw cell was firing spontaneously at a rate of 15-20 spikes per second and readily responded to DL-homocysteic acid (b, f, h, j, l, n, p). Barrel 1 was used for brief test ejections of acetylcholine (a, d, g, i, k, m and o) and barrel 2, initially used for the same purpose (c, d, e), was subsequently used to administer acetylcholine for 170 seconds. With the currents which were used, acetylcholine was approximately equally effective when passed out of these two different barrels, (compare a and c). Furthermore, simultaneous ejection from the two pipettes (d) produced a higher firing frequency than did ejection from either barrel alone (a and c), and, in addition, summation was also observed with acetylcholine and DLH (compare e with c and f).

During the prolonged administration of acetylcholine, the initial firing frequency was not maintained and the response gradually faded. In contrast with the practically unaltered effectiveness of DLH (compare e with h, j, l), the excitant action of test doses of acetylcholine was reduced (compare d, g, i and k). Furthermore, during the recovery phase, the amino acid responses (n and p) were unaffected but the first test with acetylcholine (m) showed that the sensitivity of the cell was still diminished. This Renshaw cell was thus specifically desensitized to acetylcholine and the results indicate that the fade of response during the prolonged administration of the excitant was also a specific process.

In some experiments, particularly when the rate of firing produced by the continuous administration of acetylcholine was high, the desensitization appeared to be non-specific. This non-specific effect presumably masked any specific desensitization which may have been present. Fig. 43 A shows a typical response to an administration of acetylcholine. During the control period, when acetylcholine was administered by brief current pulses (about 8 sec) at intervals of about 8 sec, the responses remained at a constant level. Thus, it was possible to employ brief ejections to follow the recovery after a prolonged administration. The initial high rate of firing was not maintained during a prolonged application of acetylcholine (Fig. 43 A), and the frequency declined over a period of about 1 min to reach a plateau. Subsequent testing showed that recovery from the desensitization took about 1 min. During prolonged applications, the rate of decline from the initial rate of firing was greater with high than with low concentrations of acetylcholine. The final plateau attained during the ejection increased with the amount of acetylcholine ejected, although the increase was not as large as it was for the initial frequency (Fig. 44). The prolonged application of acetylcholine also depressed the response to test doses of DL-homocysteic acid (Fig. 43 C) and the recovery followed a time-course similar to that of the recovery of acetylcholine (Fig. 43 A). The non-specific nature of this depression is also shown in Fig. 43 B and D, in which prolonged administration of the amino acid reduced the effect of subsequent brief ejections of the amino acid (B) or of acetylcholine (D).

Carbamylcholine and nicotine were more potent excitants of Renshaw cells than was acetylcholine (see section V B). Both substances produced non-specific desensitization but, when the rate of firing remained low, a specific depression of the response to acetylcholine was sometimes observed. The prolonged specific desensitization to acetylcholine after a prolonged electrophoretic ejection of carbamylcholine is shown in Fig. 45. In this experiment, control responses to acetylcholine (8 nA) and DLH (20 nA) are shown in Fig. 45 A. Record B was obtained 1 min after carbamylcholine began to diffuse from the micro-pipette; the firing rate increased from zero to about 30/sec and the response to a brief test dose of acetylcholine was now smaller than the response to DLH, even though the responses were similar during the control period (Fig. 45 A). When the ejection was terminated at the end of record B, the effect of acetylcholine partially recovered over a period of about 4 min.

The action of carbamylcholine in another experiment is illustrated in Fig. 46. In this experiment, diffusion of carbamylcholine from the electrode was prevented by a retaining current (-8 nA). When the retaining current was removed (Fig. 46 A), allowing the carbamylcholine to diffuse from the micro-pipette, the rate of firing rose to a maximum and then declined slightly to a plateau. Subsequent applications of acetylcholine (17 nA) produced smaller responses than those in the initial control period. This depression was far more marked and prolonged when carbamylcholine was actively ejected with a current of 5 nA (Fig. 46 B). Such a prolonged depression was

never observed when acetylcholine was administered for even longer periods.

Depression of the response to acetylcholine was observed with other cholinomimetics even in the absence of excitation and, with such compounds, the desensitization was presumably specific. The depressant effect of dl-muscarine on the responses to simultaneously administered acetylcholine is shown in Fig. 47 A. In contrast, the closely related compound, D(-) muscarone, excited the cell and depressed the subsequent responses to acetylcholine (Fig. 47 B). Unfortunately, the action of D(-) muscarone on amino-acid firing has not been tested and it is not possible to say whether the illustrated depressant effect was specific.

Long chain choline esters, such as laurylcholine and palmitylcholine, did not excite Renshaw neurones, but nevertheless reduced the effectiveness of acetylcholine, presumably by a specific mechanism. In low concentrations a compound of intermediate chain length, caproylcholine, decreased the sensitivity of Renshaw neurones to the action of acetylcholine but not to that of an amino acid (Fig. 48); in higher concentrations caproylcholine excited Renshaw cells.

The effects of acetyl-β-methylcholine on Renshaw cells were quite variable and complex. In most experiments, during the administration of acetyl-β-methylcholine, the cell either did not fire (Fig. 49) or it fired at a low frequency compared with the frequency which was attained soon after the ejection ceased (Fig. 30, 33). In the former experiments, the responses produced by acetylcholine,

ejected during the application of acetyl-β-methylcholine, were depressed (Fig. 49) but the effect of DL-homocysteic acid was enhanced.

The experiments showed that the effects of cholinomimetics on Renshaw cells were complex, consisting of excitation, specific desensitization and non-specific desensitization. It was therefore of interest to determine whether they also had depressant actions on spinal interneurones which are not excited by cholinomimetic substances (Curtis et al., 1961).

b) Depressant effects of cholinomimetics on spinal interneurones

Acetylcholine, carbamylcholine, nicotine, caproylcholine and γ-propiobetaine methyl ester depressed the amino acid-induced firing of spinal interneurones. However, the magnitude and duration of the effects on different cells were variable, and rarely as prolonged as those produced by carbamylcholine in the experiment illustrated in Fig. 50. In this experiment, the interneurone was fired by repeated ejections of DL-homocysteic acid. During the administration of acetylcholine (Fig. 50 A), with an ejecting current of 28 nA, the effect of the amino acid was at first reduced, and then despite a maintained ejection of acetylcholine, the firing frequency began to return to the control level. The action of carbamylcholine was more marked and prolonged, even when this substance was allowed to diffuse passively from the electrode by removing the retaining voltage (CARB 0 nA in Fig. 50 B). The difference in the time course of the onset of these effects may be due, in part, to different rates of administration, since ACh was actively ejected and carbamylcholine merely diffused from the micro-pipette. When carbamylcho-

line was ejected near the same cell with a current of 20 nA (Fig. 51), the spike potentials, superimposed on the focal synaptic potentials produced by stimulation of the sural nerve, were suppressed (Fig. 51 B). After the ejection was terminated, spike potentials reappeared (Fig. 51 C).

Triethylamine (see section IV D) and pyridine also depressed the excitant effects of amino acids on spinal interneurones, on cells in the cerebral cortex and on Renshaw cells. It is therefore possible that the mode of action of these substances was similar to that of acetylcholine, carbamylcholine and nicotine on spinal interneurones.

These experiments on interneurones demonstrate that cholinomimetic substances may depress the excitant action of amino acids, even though the cholinomimetics do not themselves produce excitation. The mechanism by which this depression is produced is unknown. However, it could be similar to the non-specific desensitization observed on Renshaw cells, or alternatively, the depression of amino acid induced excitation may be produced by a different mechanism, for example, a mechanism of action similar to that of inhibitory transmitters cannot be excluded.

Since acetylcholine produced both a specific and a non-specific desensitization of Renshaw cells, it was of interest to determine whether desensitization phenomena also occurred when the excitatory transmitter was released upon these cells by synaptic activation.

c) Desensitization produced by synaptic action

When a test volley in the ventral root was preceded at various intervals by a conditioning volley, the number of spikes in the initial response to the test volley was progressively reduced as the interval between volleys was increased from 12 to 30 msec. Fig. 52 B,D). A maximum reduction occurred when the interval between volleys was between 30 and 60 msec. As the interval was lengthened further, the number of spikes slowly increased and returned to the control value when the volley interval was about 1sec. The volley-interval for which there was a maximum depression of the initial response to the test volley (Fig. 52 D) corresponded to the time at which the initial response to a single stimulus had decreased to zero and was succeeded by the pause (Fig. 52 C). Throughout the whole range of intervals shown in Fig. 52 D there was no change in the late response which followed the test volley.

This coincidence of the time at which the initial response to a single stimulus had decreased to zero (Fig. 52 C) and the time interval for which the depression of the test response was maximal (Fig. 52 D), suggests that the decline in firing frequency during the initial response and the subsequent pause are inter-related and caused by the same phenomenon, namely desensitization; the reduction in the response to a test volley after a conditioning volley in the ventral root may also be partly due to transmitter depletion.

Desensitization following a ventral root volley was also more directly demonstrated in three ways. Firstly, when brief pulses

(about 50 msec duration) of acetylcholine were ejected from the micropipette after a single orthodromic (ventral root) stimulus, the response to acetylcholine was reduced. Secondly, when a single stimulus was applied to the ventral root during the continuous administration of acetylcholine, which raised the background firing frequency to about 50/sec, the pause was still apparent. Under these conditions the number of spikes in the initial response was reduced, presumably because the administered acetylcholine decreased the effect of the transmitter released by nervous stimulation. Thirdly, the effect of a pulse of acetylcholine (duration, about 5 sec) was decreased following tetanic stimulation (100/sec for 3 sec) of the ventral root; following tetanic stimulation, the pause is prolonged (see Fig. 41). Similar experiments with DL-homocysteic acid showed that the desensitization following ventral root stimulation was not specific for acetylcholine.

This non-specific desensitization does not appear to be the consequence of the high frequency spike discharge since the electrophoretic application of dihydro-β-erythroidine reduced the number of spikes in the initial response to a single ventral root stimulus from about 35 to 2 spikes, but it did not affect the duration of the pause.

Thus, after a stimulus to the ventral root there follows a non-specific desensitization to acetylcholine, which is not the result of the initial high frequency discharge. This desensitization would, in part, explain the decline in firing frequency during the

initial response. This decline is presumably also partly due to diffusion of transmitter away from the receptor sites (Eccles and Jaeger, 1958), and to inactivation of acetylcholine by acetylcholinesterase.

d) The actions of anticholinesterase agents

Anticholinesterases, under certain conditions, prolong the duration of the initial response (Eccles et al., 1954, 1956; Curtis and Eccles, 1958b; Curtis et al., 1961). However, it is not certain that this prolongation is entirely related to inactivation of cholinesterase, since most anticholinesterase agents also had excitant effects on Renshaw cells. Fig. 53 demonstrates the effect obtained with neostigmine in one experiment. In this experiment, the neostigmine in the micro-pipette was dissolved in 165 mM NaCl solution to give a final concentration of 10 mM. Thus, only a relatively small amount of neostigmine would actually have been passed out of the electrode. Typically, the excitant action of neostigmine was prolonged, as in Fig. 53. A similar, prolonged effect was also obtained after the electrophoretic ejection of physostigmine, but edrophonium had only a brief action. The excitant effect of neostigmine was partially depressed by the ejection of dihydro-β-erythroidine in amounts sufficient to abolish completely the response to acetylcholine. Thus, the excitation may be the consequence of inactivation of cholinesterase which could cause spontaneously released acetylcholine to be more effective. This interpretation explains why the effect of anticholinesterase agents was partially blocked by

dihydro-β-erythroidine. However, it is possible that the anticholinesterase agents react directly with acetylcholine receptors, which would also explain the partial depression of their excitant actions by dihydro-β-erythroidine. This postulated direct excitant effect of anticholinesterase agents is in accordance with the interpretation of their effects at other sites (see review by Werner and Kuperman, 1963).

ii) Discussion

These experiments have demonstrated that a number of cholinomimetics, when administered electrophoretically to Renshaw cells in the spinal cord of the cat, desensitize these cells to subsequent doses of excitants, a phenomenon described in theoretical terms by Paton (1961).

As a consequence of this theory, the following events may be predicted when cholinomimetic substances react with suitable receptors. At the instant when a molecule of drug reacts with a receptor, the process leading to excitation of the cell is initiated. When sufficient numbers of receptors are occupied simultaneously, the neurone may fire (i.e. the rate of combination with receptors has reached a certain critical value). As the number of receptors occupied at a particular instant increases, so the rate of combination will fall, since fewer receptors are available for occupation. Therefore, the degree of excitation will diminish. If the rate of dissociation is much slower than the rate of association, the critical rate of combination may not be reached, and the drug will tend to block the

interaction between the receptor and another cholinomimetic agent which has rate constants suitable for causing marked excitation. The effect of excitant substances (such as amino acids), reacting with a different receptor, will be unaffected. This type of action is exerted on the Renshaw cell by specific blocking agents such as dihydro-β-erythroidine (see section V B). It also occurred with long chain choline esters (laurylcholine and palmitylcholine), which did not produce excitation, and a similar, specific desensitization was demonstrated with caproylcholine, but this compound also had weak excitant effects.

Potent excitants, such as acetylcholine, carbamylcholine and nicotine, showed a decrease in the response with continued exposure to the excitant, which is predicted from Paton's theory. In part, this desensitization was specific and this could be demonstrated with low concentrations of the excitants. With higher concentrations, and higher rates of firing, the desensitization appeared to be non-specific, since the effectiveness of DL-homocysteic acid was also reduced.

In many respects, the desensitization produced by acetylcholine on Renshaw cells is similar to that found at the motor end-plate on skeletal muscle (Thesleff, 1955a, b; 1956, 1959; Thesleff and Katz, 1957; Axelsson and Thesleff, 1958). These investigators were able to follow the process of desensitization by an intracellular recording technique, which was not feasible in experiments on Renshaw cells. Thesleff (1956) concluded that the desensitization at the end-plate was not due to depolarization or to a change in membrane

conductance, but was due to receptor-desensitization, similar to that produced by d-tubocurarine.

Unfortunately, there was no excitant other than acetylcholine which could be used at the end-plate to check the receptor specificity of the desensitization. In this respect, the experiments on Renshaw cells have yielded additional information. Here it was shown that specific desensitization could be produced by acetylcholine and other cholinomimetics. In addition, non-specific desensitization occurred with large concentrations of potent excitants and was also seen after synaptic activation.

Non-specific desensitization could arise in a number of ways. For example, it could be due to the depletion of some intra-cellular component that is essential for the maintained activity of the neurone. If this were so, then the desensitization should be related to the magnitude of the preceding spike discharge. However, dihydro-β-erythroidine greatly reduced the number of spikes in the initial response to a ventral root volley but the period of desensitization (i.e. the pause) which followed it was unaffected. This observation also makes it unlikely that the desensitization was due to a after-hyperpolarization of the cell membrane.

There was no change in the size or shape of the spikes during the period of desensitization produced by the electrophoretic administration of excitant cholinomimetic substances and the terminal spikes in the initial response were quite normal. Furthermore, the desensitization caused by a single ventral root volley was maximal

30-60 msec after the stimulus and at this time the membrane potential has returned to the resting state (Eccles et al., 1961). Therefore, excessive depolarization or a disturbance of the spike generating mechanism is unlikely to be the cause of the non-specific desensitization, although they may be contributory factors during the first few msec of the ventral root discharge when irregularities in spike size, shape and frequency were observed (see also, Eccles et al., 1961).

A purely speculative explanation of the possible mechanisms by which the various types of desensitization could be produced is illustrated in Fig. 54. This explanation assumes that there may be 'intermediate' stages somewhere along the hypothetical pathway linking receptor occupation with the final excitatory pathway: this concept of 'intermediate' stages is not new (see Thesleff and Katz, 1957). The figure is not intended to depict any spatial relationships between receptors, but merely shows that intermediate MN or S-receptors may be common to more than one pathway. There is really no conclusive evidence for the presence of an 'intermediate' receptor, unless it is similar to the receptor protein, isolated from electric tissue by Ehrenpreis (1960, 1962a,b, 1963a, b, 1964), which is probably not identical with the 'physiological acetylcholine receptor' (Ehrenpreis, 1962a, b, 1963b).

Specific desensitization, involving only the M, N or A-receptors, could occur by occupation (with appropriate kinetic constants) of the receptors. This is the postulated mode of action of atropine, dihydro-β-erythroidine and long chain choline esters and of excitant cholinomimetics in low concentrations. It is perhaps

relevant to note that no specific antagonist for amino acid excitation has yet been discovered. Thus Path Z is probably unnecessary at present, since there is no evidence to show that the amino acid receptor is not common to a number, if not all, excitatory processes controlled by chemical transmitter substances, and the intermediate receptor S may therefore be identical with the amino acid A-receptor.

Non-specific desensitization to the actions of acetylcholine and of an amino acid could be produced in different ways. Firstly, it could be the consequence of excessive excitation; this has already been discussed, and an attempt was made to exclude the possibility. Secondly, occupation with appropriate kinetic constants, and therefore desensitization of an S-receptor by high concentrations of cholinomimetics, could lead to a reduction in the effectiveness of both acetylcholine and an amino acid, and is preceded by excitation due to interaction with the N-receptor. This idea incorporates the not unreasonable assumption that the occupation of S-receptors by cholinomimetics leads mainly to desensitization due to the different kinetics of the reaction, whereas interaction with N-receptors leads mainly to excitation. This type of action could explain the non-specific desensitization produced by acetylcholine and synaptic activation without the need to postulate that desensitization is the direct consequence of the excitation process. This mechanism could also explain the ability of cholinomimetic agents to depress the amino acid induced firing of interneurones, which are not excited by cholinomimetics, and the spontaneous firing of many neurones in the central

nervous system (Bradley and Wolstencroft, 1962; Von Baumgarten, Bloom, Oliver and Salmoiraghi, 1963; Salmoiraghi and Steiner, 1963; Spehlmann, 1963; Bradley, Dhawan and Wolstencroft, 1964; Krnjevic, Randic and Straughan, 1964).

Acetyl-β-methylcholine excited Renshaw neurones mainly by an interaction with muscarinic receptors (section V B). However, the rate of firing often did not increase greatly during the application, but increased when the ejection was terminated. During the application, the excitant effect of acetylcholine, which mainly reacts with nicotinic receptors (section V B), was reduced and the excitant effect of an amino acid, which interacts with other receptors, was enhanced. One explanation of the selective depression of the response to acetylcholine is that, besides activating muscarinic receptors, acetyl-β-methylcholine also reacted with and desensitized nicotinic receptors. There was some evidence for an interaction with nicotinic receptors but this interpretation does not explain why the firing induced by acetyl-β-methylcholine did not increase markedly until the ejection was terminated. Since the effect of the amino acid was enhanced during the application of acetyl-β-methylcholine, the depression of firing was not due to a non-specific depression of all excitatory processes.

The hypothesis illustrated in Fig. 54 also offers an explanation of this complex action of acetyl-β-methylcholine. Interaction with M-receptors or N-receptors leads to excitation which may be antagonized by atropine or dihydro-β-erythroidine (section V B). However, if the substance were also to interact with the intermediate

MN-receptor in such a way that occupation resulted in blockade, then excitation due to occupation of M or N-receptors would be depressed but the effect of an amino acid would not be reduced. If blockade of the MN-receptors were to decrease at a faster rate than activation of the M-receptors when the ejection is terminated, then excitation would supervene, as in the experiments with acetyl-β-methylcholine.

Axelsson and Thesleff (1958) and Thesleff (1959) suggested that desensitization may be an important factor in repetitive stimulation of motor nerves to muscles. The experiments on Renshaw cells have shown that desensitization may also be important in determining the rate at which the frequency of firing decays after a single stimulus applied to the ventral root. The phenomenon may also partly explain the typical time course of the excitatory postsynaptic potentials (Eccles et al., 1961). Presumably, inactivation of acetylcholine by acetylcholinesterase is also important but it is difficult to assess its significance because the interpretation of the actions of anticholinesterase agents is complicated by their direct excitant effects.

VI) CONCLUDING REMARKS

The subject matter of this thesis has ranged over a number of topics but a central theme concerned the role of acetylcholine in synaptic transmission in the central nervous system. It was shown that the major cholinomimetic substance present in nerve-terminals was acetylcholine and that, in contrast with a number of other substances, with the possible exception of substance P, acetylcholine was the only substance which was specifically located in nerve-terminals. The methods currently employed for studying the subcellular distribution of substances which may have transmitter functions are relatively gross. Further advances in the elucidation of cholinergic mechanisms at particular sites may come from histochemical studies in conjunction with studies employing electron-microscopic techniques.

Pharmacological studies on central neurones have shown that many neurones in the nervous system react to acetylcholine but conclusive evidence that it is a transmitter is lacking, except at the synapses formed by the terminals of motor-axon collaterals on Renshaw cells. It is possible that acetylcholine may not only act as a synaptic transmitter which effects the rapid transfer of nervous information across synapses. It may also have a local hormonal action which controls the overall activity of a particular group of neurones.

Both muscarinic and nicotinic receptors were found to be present on Renshaw neurones. It was suggested that the nicotinic receptors were associated with the short term effects of synaptic

transmission, whereas the muscarinic receptors were concerned with more prolonged effects. A speculative hypothesis was proposed that, at all sites at which cholinergic transmission occurred in vertebrates, the subsynaptic cholinoceptive receptors were nicotinic in type and that muscarinic receptors were extra-synaptic. Thus, it is possible that the muscarinic receptors are those involved in the long term, local hormonal effects of acetylcholine.

In section (V D) another aspect of the pharmacological effects of acetylcholine on central cholinoceptive neurones was considered. It was shown that desensitization may be an important additional physiological mechanism by which the duration of transmitter-action is controlled. Until other transmitters have been identified, it is not yet possible to say whether this action has universal applicability to all types of synaptic transmission. However, receptor desensitization may now be added to the list of processes by which the action of a transmitter may be limited in time.

A subsidiary development in this thesis included attempts to identify the transmitter which depolarizes presynaptic terminals and causes 'presynaptic inhibition': these experiments eliminated some of the possibilities but gave no indication of the nature of the substances which cause presynaptic inhibition.

Another avenue which has been explored was the possibility of applying purified brain extracts electrophoretically to single neurones. These experiments showed that the method was technically feasible, but they yielded no new information concerning the nature

of central synaptic transmitters. Further refinements in the technique may prove to be fruitful in shedding new light on the substances which effect synaptic transmission in the central nervous system.

Drugs used

Acetylcholine bromide
Acetyl-DL-carnitine
Acetylhomocholine chloride
Acetyl-β-methylcholine chloride
Acetylthiocholine iodide
nor-Adrenaline bitartrate hydrate
γ-Aminobutyrylcholine chloride
γ-Amino-n-butyric acid
Arecoline hydrobromide
Atropine sulphate
Bromolysergic acid diethylamide
γ-Butyrobetaine methyl ester bromide
n-Butyrylcholine iodide
Carbamylcholine chloride
Caproylcholine bromide
Cetyltrimethylammonium bromide (Cetavlon)
α-Chymotrypsin (Sigma)
Dihydro-β-erythroidine hydrobromide
Edrophonium chloride
Glutamic acid, sodium salt
DL-Homocysteic acid, sodium salt
5-Hydroxytryptamine creatinine sulphate
Hyoscine hydrobromide
Hexamethonium bromide
Iproniazid (1-isonicotinoyl-2-isopropylhydrazine phosphate)
Laurylcholine bromide
Mepyramine maleate
dl-Muscarine chloride
D(-) Muscarone chloride
Neostigmine bromide
Nicotine hydrochloride
Palmitylcholine bromide
Pentobarbitone sodium
Picrotoxin
Physostigmine (eserine) salicylate
Pilocarpine hydrochloride
Procaine hydrochloride
Propionylcholine bromide
γ-Propiobetaine methyl ester chloride
Strychnine hydrochloride
Substance P
Succinylcholine bromide
Tetraethylammonium bromide
Tetramethylammonium bromide
Tryptamine hydrochloride
d-Tubocurarine chloride

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TABLE 1. Fractionation of brain homogenates: Method (a)

Fraction P1 was separated in an MSE refrigerated centrifuge (Major). Subsequent fractionations were carried out in the No. 30, 40, or SW 39 rotor of the Spinco Model L ultracentrifuge.

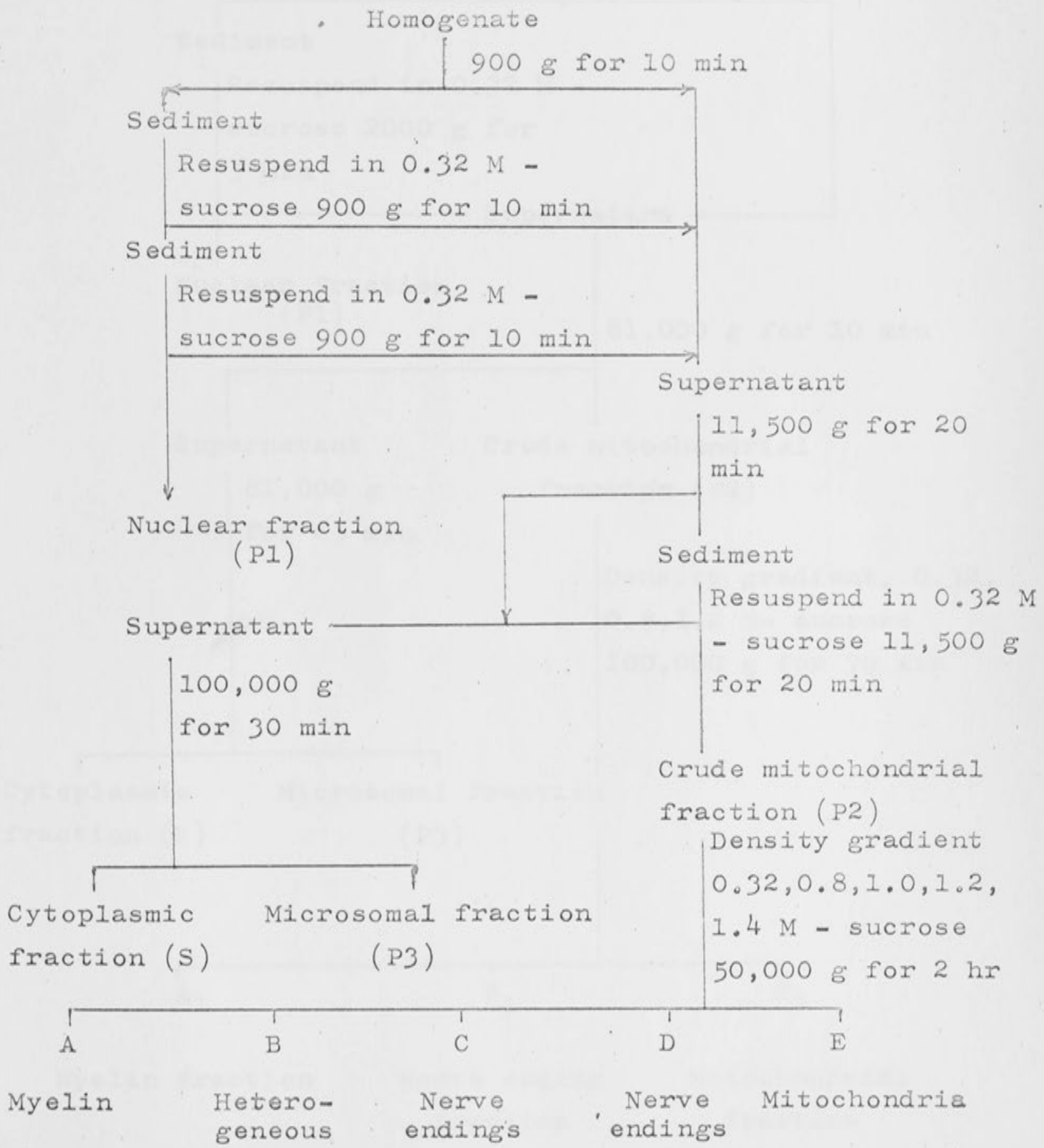

TABLE 2. Fractionation of brain homogenates: Method (b)

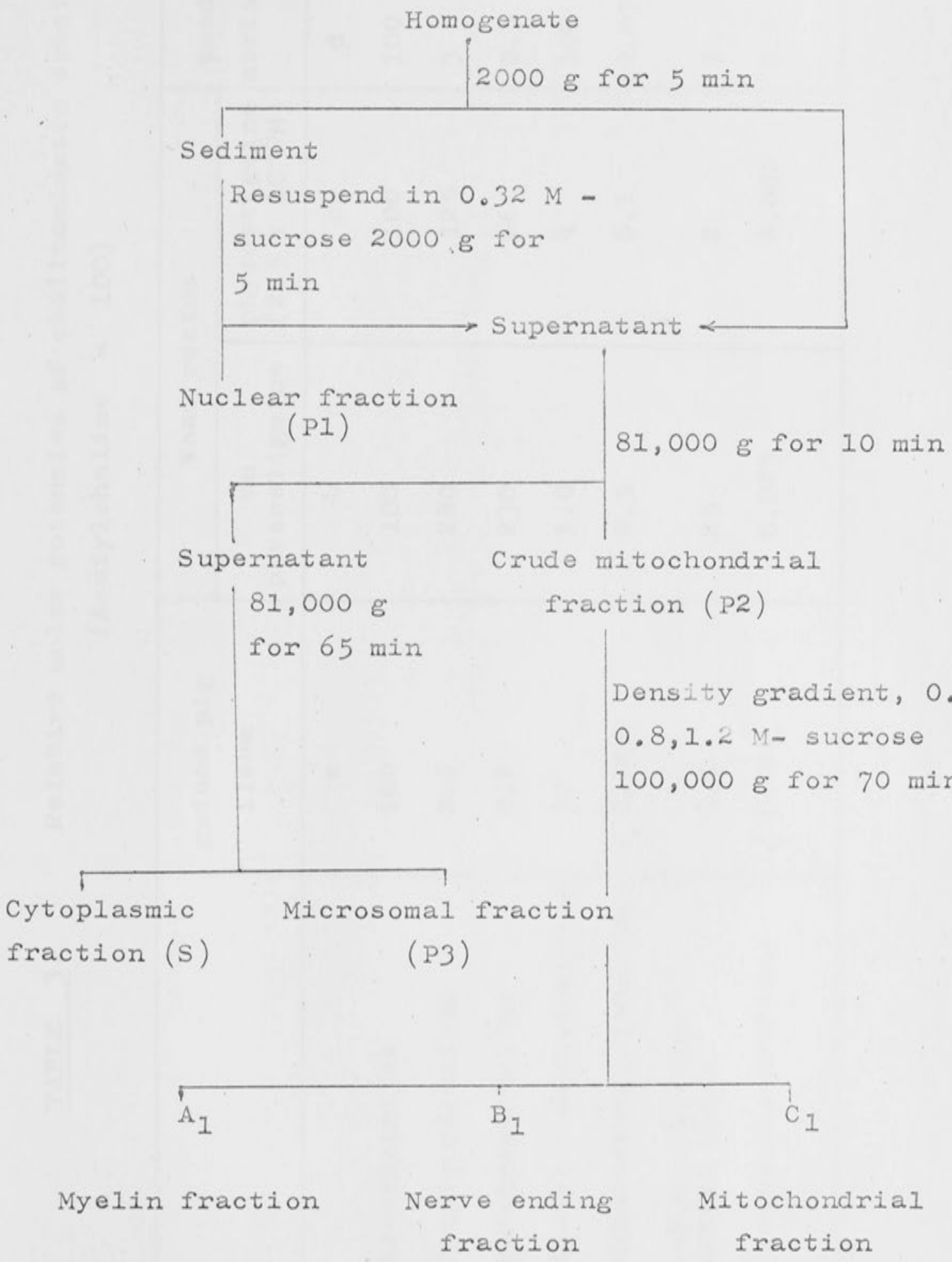

TABLE 3. Relative molar potencies of cholinomimetic substances (Acetylcholine = 100)

	guinea pig ileum	toad rectus		toad atria	guinea pig atria
		no physostigmine	physostigmine (2.5×10^{-5}M)		
	a	b	c	d	e
Acetylcholine	100	100	100	100	100
Propionylcholine	2.6	280	125	3	0.6
n-Butyrylcholine	0.2	230	66	0.3	0.1
Acetyl-β-methylcholine	37	1.0	4	100	25
γ-Aminobutyrylcholine	< 0.025	0.5	0.1	<0.05	< 0.03
γ-Butyrobetaine methyl ester	6.0	25	2	7	10
Acetyl-DL-carnitine	< 0.001	0.025	0.002	0.1	0.003

TABLE 4. Indices of discrimination of cholinomimetic substances

(Acetylcholine = 1)

Preparations*	Propionyl-choline	n-Butyryl-choline	Acetyl-β-methyl-choline	γ-Amino-butyryl-choline	γ-Butyrobe-taine methyl ester	Acetyl-DL-carnitine
b/a	108	1150	37	>20	4.3	25
c/a	48	330	9.3	>4	3	>2
d/a	1.2	1.5	2.7	-	1.1	>100
e/a	4.3	2	1.5	-	1.7	>3
b/c	2.2	3.5	4	5	13	12.5
b/d	93	770	100	>10	3.7	4
b/e	470	2300	25	>16	2.6	8.3
c/d	42	220	25	>2	3.5	50
c/e	210	660	6	>3	5	1.5
d/e	5	3	4	-	1.4	33
Highest index	470(b/e)	2300(b/e)	100(b/d)	>20(b/a)	13(b/c)	100(d/a)

*The letters refer to the preparations in Table 3 and the indices are expressed as for example, b/a or its reciprocal a/b so as to give values greater than 1.

TABLE 5. Relative molar potencies of cholinomimetic substances (ACh = 100)

	Salt	Nicotinic potency 1	2	3	Muscarinic potency 4	5	6	Renshaw cells 7	8
Nicotine	HCl	700	30-400	700	0.04			++++	>1000
Acetylthiocholine	I	500	50	800	0.06			+(+)	
γ-Propiobetaine methyl ester	Cl	320	650	2400	30			++++	>400
Carbamylcholine	Cl	200	120	330	27			++++	600-3000
Tetramethyl-ammonium	Br	170	10	70	0.08			++++	>1000
Acetylcholine	Br	100	100	100	100	100	100	++	100
Propionylcholine	Br	96	280	380	2.6	0.6	3	+	50
n-Butyrylcholine	I	73	230	270	0.2	0.1	0.3	+	30
Caproylcholine	Br	50	250		0.5			+/block	
Acetylhomocholine	Cl	12	20	680	2.2			+	
Succinylcholine	Br	10	430	10^4	<0.01			+	60
Acetyl-β-methyl-choline	Cl	<20	1	4	37	25	100	+	3-30
Arecoline	HBr	<10			21			+	<20

continued

TABLE 5. (continued)

	Salt	Nicotinic potency 1	2	3	Muscarinic potency 4	5	6	Renshaw cells 7	8
Pilocarpine	HCl	<10	<0.03		0.5			+	<10
D(-) Muscarone	Cl		200*					+	50
dl-Muscarine	Cl		<1*		250-400*		<100*	+	20
γ-Butyrobetaine methyl ester	Br		25		6	10	7	+++	200
γ-Aminobutyryl-choline	Cl	20	0.5	14	<0.025			+/block	
Laurylcholine	Br							block	
Palmitylcholine	Br							block	

1.) Blood pressure of atropinized cat; 2) toad rectus; 3) chick semispinalis cervicis; 4) guinea pig ileum; 5) guinea pig atria; 6) toad atria; 7) order of excitatory activity on Renshaw cells (ACh = ++); 8) potency as an excitant of Renshaw cells relative to ACh (= 100); (+/block) shows that the substance had a weak excitatory effect and depressed the action of ACh.

TABLE 6. Electrophoretic mobilities at pH 4 and chromatographic Rf values of known cholinomimetic substances

Substance	Electrophoretic mobility* (cm from origin)	Band**	Rf in buffered solvent+
Acetylcholine	5	B	0.51
Propionylcholine	5	B	0.65
n-Butyrylcholine	5	B	0.72
γ-Aminobutyrylcholine	6.4	B	0.19
Acetyl-β-methylcholine	5.2	B	0.65
γ-Butyrobetaine methyl ester	4.7	B	0.59
Acetyl-DL-carnitine	1.4	A	0.56

*Electrophoretic conditions identical with those used in the separation of the extract.

**Band is the electrophoretic fraction of the brain extract in which the various substances would be recovered if they were present.

+Solvent, triethylamine/acetic acid/water/1-butanol (33:4:8:10 by vol).

TABLE 7. Parallel assays on unpurified crude mitochondrial extracts

Preparation	Percentage of value obtained on the guinea pig ileum	
	Extract 1.	Extract 2.
Guinea pig ileum	100	100
Rat blood pressure (neostigmine)	107	-
Toad rectus (no physostigmine)	92	120 (89*)
Toad rectus (physostigmine 2.5×10^{-5} M)	-	100
Rabbit atria (physostigmine 0.25×10^{-5} M)	93	-
Guinea pig atria (no physostigmine)	-	110
Guinea pig atria (physostigmine 0.25×10^{-5} M)	-	77
Toad atria (no physostigmine)	74	51
Toad atria (physostigmine 2.5×10^{-5} M)	76	81
+Cholinomimetic activity on guinea pig ileum (µg/g brain)	0.9	1.3

*In this assay allowance was made for sensitising substances present (Feldberg and Mann, 1945).

+Expressed a µg of ACh bromide per g fresh brain weight.

TABLE 8. Subcellular distribution of ACh and nitrogen. (See Table 9 for explanation of symbols)

Column	1	2	3	4		5	6
Species	rat*	rat	rat	sheep*+		guinea pig+	guinea pig+
Method	(a)	(a)	(a)	(a)		(b)	(b)
Number of experiments	3	1	3	1		3	2
Fraction	ACh %R (RSA)	ACh %R (RSA)	N_2 %R	ACh %R	fraction	ACh %R (RSA)	N_2 %R
P1	6 (0.61)	5 (0.45)	11	12	P1	17 (0.77)	22
P2	53 (1.23)	82 (1.9)	43	38	P2	71 (1.4)	51
P3	11 (0.79)	13 (0.93)	14	17	P3	3 (1.25)	2.4
S	30	0	32	34	S	8	24
A	3 (0.25)	15 (1.25)	12	10	A_1	3 (0.31)	9.1
B+C	23 (1.5)	55 (3.7)	15	14	B_1	31 (1.4)	22
D	2.3(0.58)	4 (1.0)	4	2	C_1	0.1(0.07)	1.5
E	0.5(0.2)	4 (1.5)	2.7	1			
R/g brain	2.3 µg	0.85 µg	12.5 mg	0.19µg		0.39 µg	13.8mg
%P2	54	95	79	71		48	64

TABLE 9. Subcellular distribution of substance P

Column	1	2		3
Species	rat	sheep		guinea pig
Method	(a)	(a)		(b)
Number of experiments	4	1		3
Fraction	Substance P %R (RSA)	Substance P %R	Fraction	Substance P %R (RSA)
P1	11 (1.0)	22	P1	12 (0.55)
P2	61 (1.4)	52	P2	80 (1.55)
P3	23 (1.65)	26	P3	5 (2.1)
S	5	0	S	3
A	10 (0.84)	19	A_1	11 (1.2)
B+C	38 (2.5)	15	B_1	49 (2.2)
D	3 (0.75)	0	C_1	0.5(0.3)
E	1.6(0.59)	0		
R/g brain	37 units	32 units		61 units
%P2	86	66		76

Key to Tables 8-10

*Sucrose media contained physostigmine (2.7×10^{-5} M)

**Sucrose media contained iproniazid (1-isonicotinoyl-2-isopropylhydrazine), 1 mM

+ Unanaesthetised animals

R = total activity recovered in P1, P2, P3 and S

%P2 = percentage of activity originally present in P2 recovered in particulate subfractions A to E

RSA = relative specific activity (see page 56)

Figures in parentheses are the relative specific activities.

TABLE 10. Subcellular distribution of 5-HT. (See Table 9 for explanation of symbols)

Column	1	2		3	4
Species	rat	guinea pig		guinea pig	guinea pig
Method	(a)	(a)		(b)	(b)(Iproniazid)**
Number of experiments	4	1		1	1
Fraction	5-HT %R (RSA)	5-HT %R (RSA)	Fraction	5-HT %R (RSA)	5-HT %R (RSA)
P1	6 (0.55)	3 (0.33)	P1	5 (0.22)	9 (0.79)
P2	27 (0.62)	21 (0.62)	P2	18 (0.35)	36 (0.74)
P3	21 (1.5)	33 (1.75)	P3	6 (2.5)	27 (9)
S	46	45	S	71	29
A	6 (0.5)	13 (0.87)	A_1	12 (1.3)	6 (0.85)
B+C	21 (1.4)	12 (1.5)	B_1	8 (0.36)	12 (0.46)
D	2 (0.5)	0 (0)	C_1	0.7(0.5)	20 (14)
E	0.5(0.2)	0 (0)			
R/g brain	0.10 µg	0.10 µg		0.11 µg	0.19 µg
%P2	109	119		115	105

TABLE 11. Relative specific activities of amino acids and ACh in guinea pig brain homogenates (Method b)

	GABA		Glutamic acid		ACh	
Fraction	RSA	No.of expts.	RSA	No.of expts.	RSA	No.of expts.
P1	0.32	4	0.34	2	0.77	3
P2	0.39	4	0.55	2	1.4	3
P3	0.33	4	0.42	2	1.25	3
S	-		-		-	
A_1	0.41	2	0.36	2	0.31	3
B_1	0.32	2	0.45	2	1.4	3
C_1	0.5	2	0	2	0.07	3

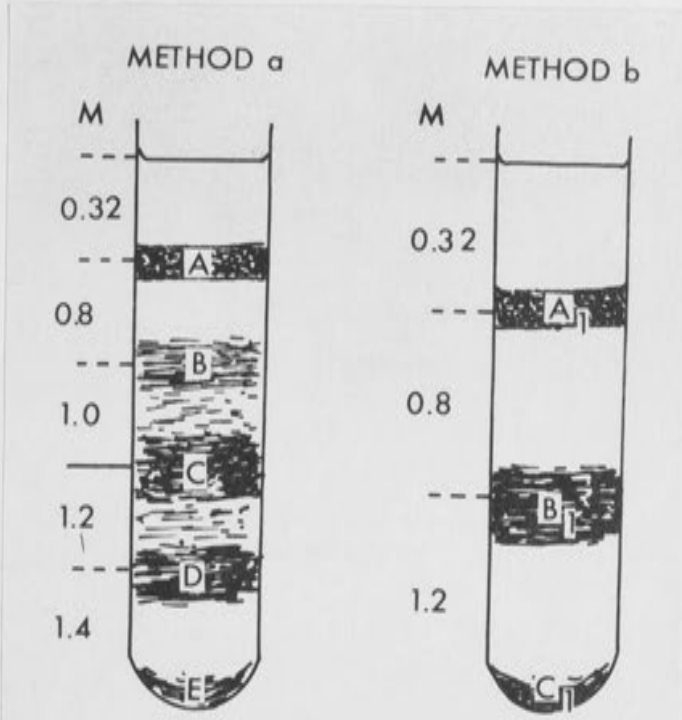

Fig. 1. Diagram showing the appearance of the centrifuge tubes after centrifuging a 'crude mitochondrial' (P2) fraction from brain over density-gradients consisting of 0.32, 0.8, 1.0, 1.2 and 1.4 M sucrose (method a, see Table 1) or 0.32, 0.8 and 1.2 M sucrose (method b, Table 2). A is a dense yellowish-white layer, containing mainly myelin fragments. Fraction B, a diffuse grey layer, merges into fraction C (the cholinergic nerve ending fraction) which consists of a tan-coloured, fairly dense layer. Fraction D (the non-cholinergic nerve-ending fraction), also tan-coloured, is separated from fraction C by a cloudy interverning region in the tube, and from fraction E (the mitochondrial fraction) by a clear region. Fraction B_1 is composed of fractions B+C+D and is separated from both the myelin fraction (A_1) and the mitochondrial fraction (C_1) by clear layers of sucrose.

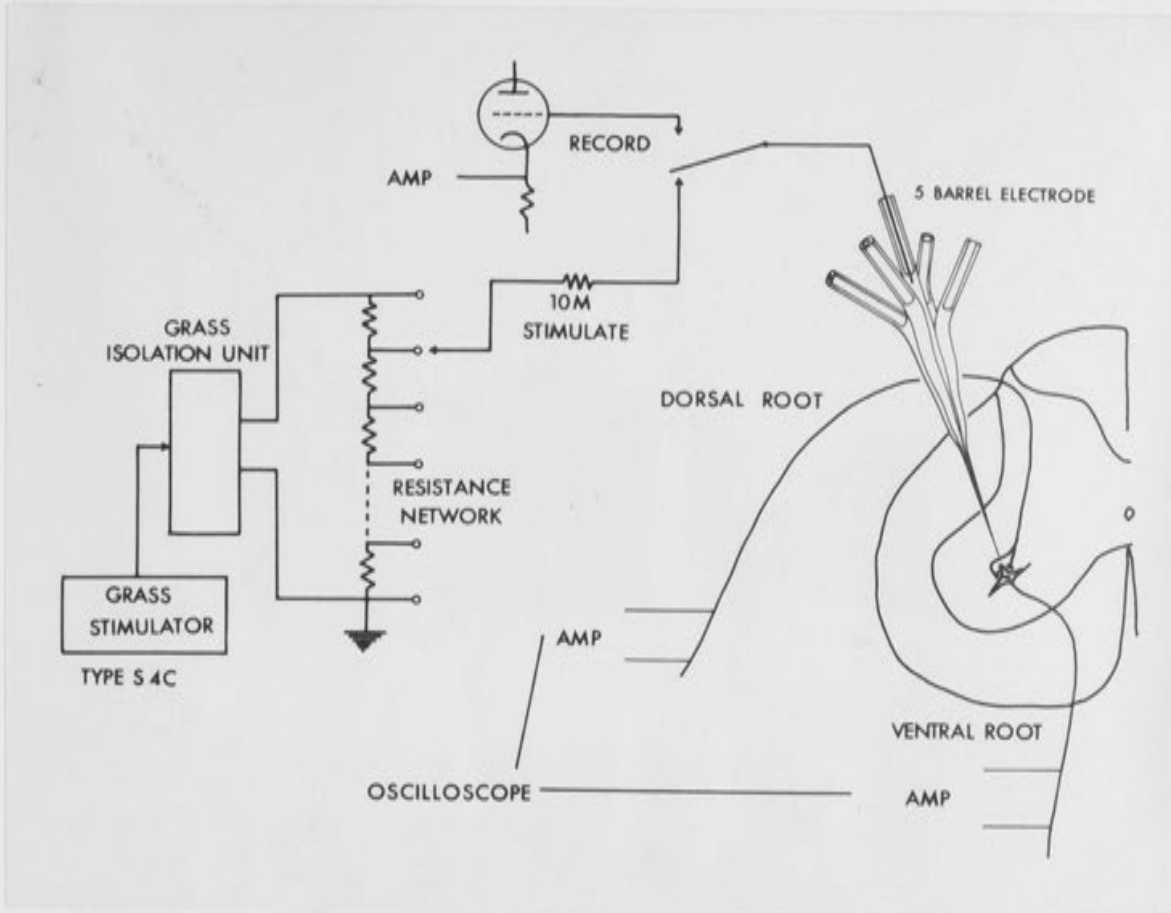

Fig. 2. Diagrammatic representation of the experimental conditions for applying drugs electrophoretically to primary afferent terminals while recording their electrical excitability. The centre barrel of the micro-electrode is used either for recording the field potentials set up by stimulating peripheral nerves, or may be connected to the output of a Grass stimulator in order to stimulate the terminals. Action potentials, recorded from peripheral nerves or from the ventral roots, are displayed on an oscilloscope.

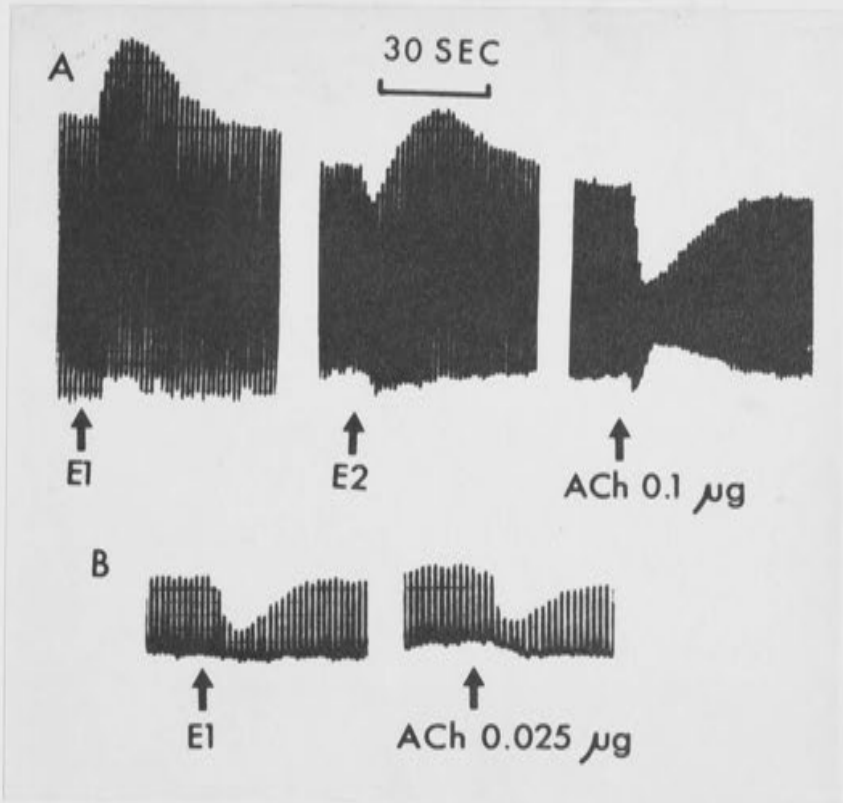

Fig. 3. (A). Contractions of isolated perfused toad's heart. E1, 0.05 ml of crude P2-extract. E2, 0.2 ml of crude extract.

(B). Same heart but ventricles removed. E1, 0.05 ml of crude extract.

Note the complex effect of the extract on the whole heart and the simpler, ACh-like depressant action on atria. Time, 30 sec.

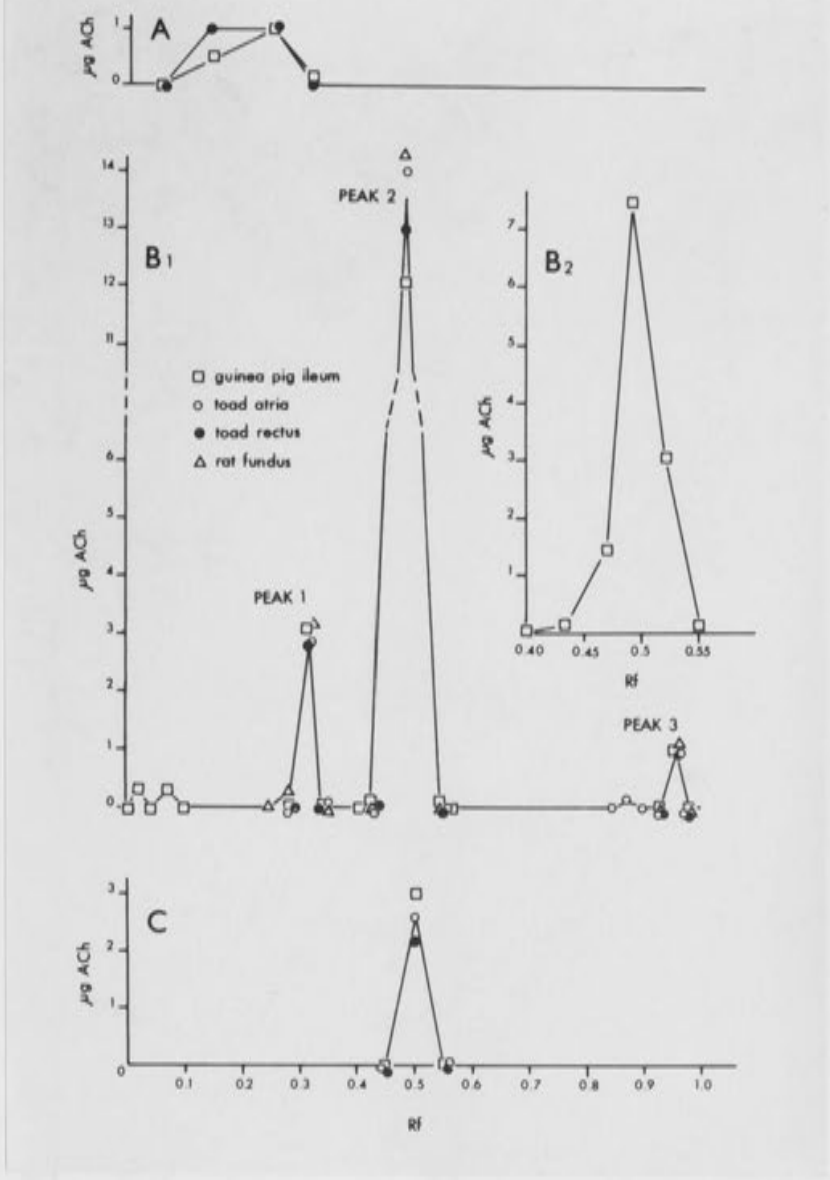

Fig. 4. Distribution of biological activity along chromatograms of cationic components of a crude mitochondrial extract, subjected to previous electrophoretic separation at pH 4. (A), Fraction A. (B_1), Fraction B. (B_2), Peak 2 of fraction B in more detail. (C), Fraction C. Fractions D and E were completely inactive. In B_1, peak 2 is the total activity of the three fractions corresponding to Rf 0.47, 0.49 and 0.52 in Fig. $3B_2$.

The ordinate is the total activity of each eluate expressed as µg of ACh bromide. Note the break in the ordinate scale in B_1. All activity except that of fraction A was attributed to ACh (see text). (□), guinea pig ileum, (O), toad atria, (●), toad rectus treated with physostigmine, (△), rat fundus. Zero activity is indicated only at the base of each peak.

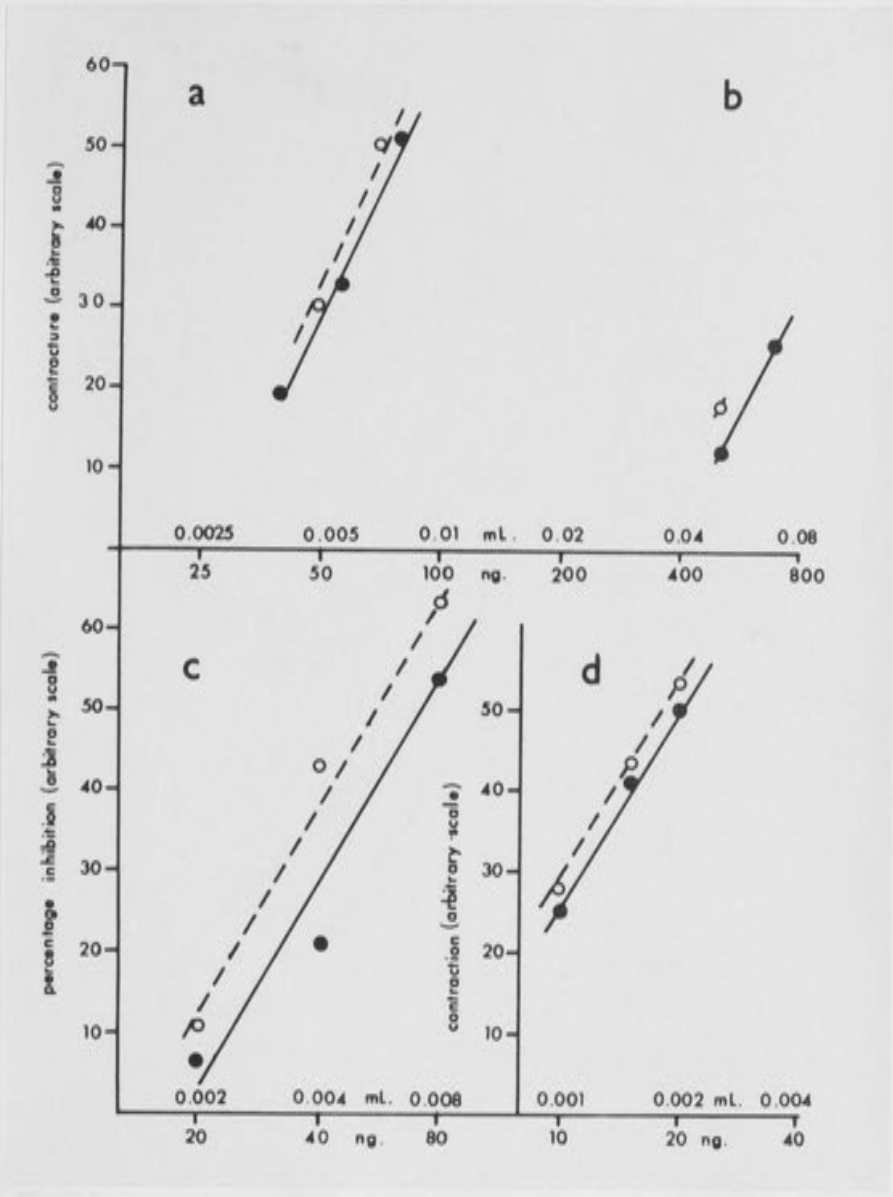

Fig. 5. Parallel assays of an active eluate from fraction B (peak 2, Fig. 4 B_1) on toad rectus abdominis (a) treated with physostigmine (2.5×10^{-5}M) and (b) untreated with physostigmine in a 1 ml bath, (c), toad atria in a 1 ml bath, (d), guinea pig ileum in a 2 ml bath. (O), eluate. (●), ACh. The abscissae represents the amount of ACh bromide in ng (below the line), or the amount of the eluate in ml (above the line). Note the similar dose-response curves on (a), (c), and (d), and equal potentiation of the effects of both the eluate and ACh by physostigmine on the toad rectus, (compare (a) with (b)).

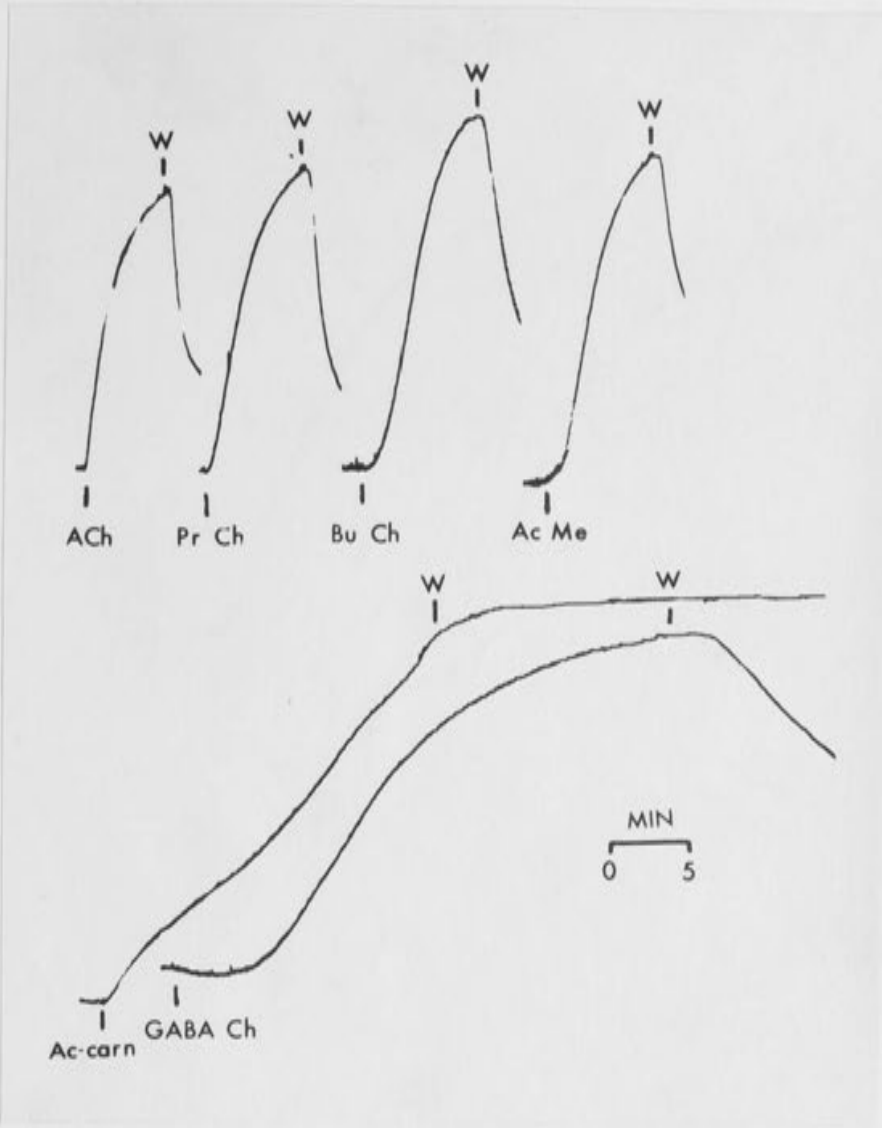

Fig. 6. Toad rectus abdominis. Contractures to acetylcholine (ACh), 0.7 μg, propionylcholine (Pr Ch) 0.5 μg, n-butyrylcholine (Bu Ch), 0.5 μg, acetyl-β-methylcholine (Ac Me) 140 μg, γ-amino-butyrylcholine (GABA Ch), 75 μg and acetyl-DL-carnitine (Ac-Carn) 4 mg. W = wash. 1 ml bath. Time - 5 minutes.

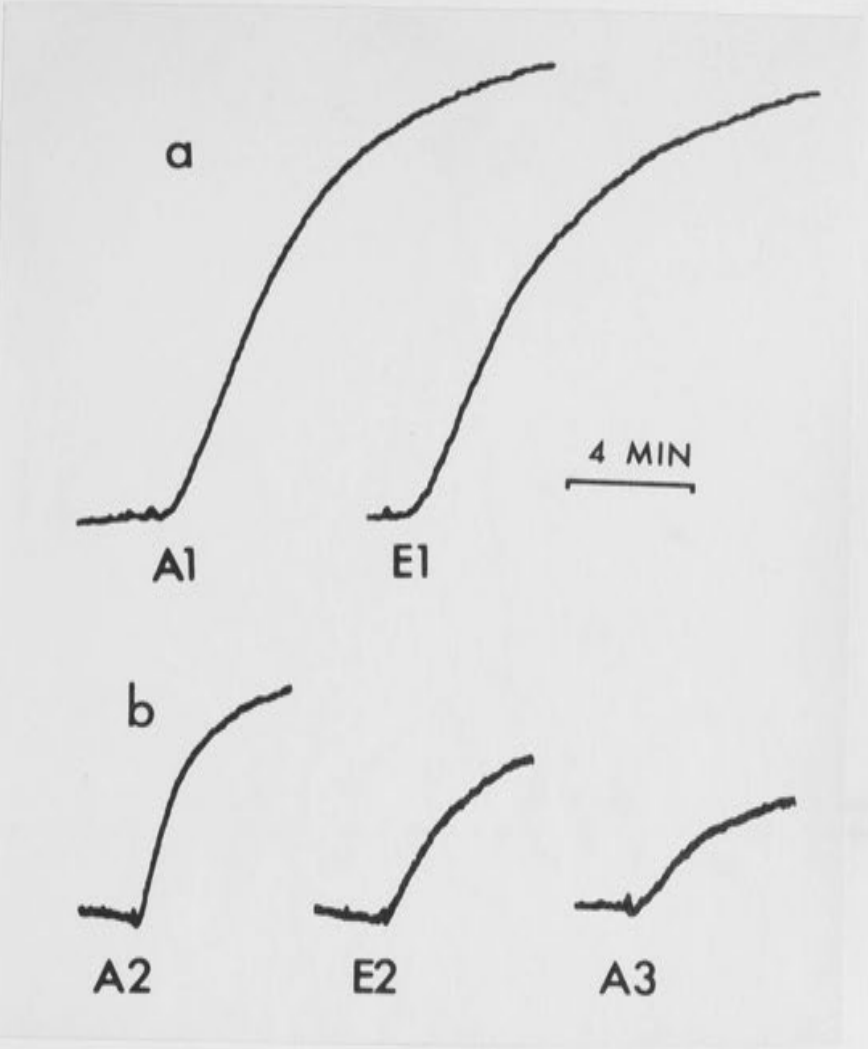

Fig. 7. Toad rectus abdominis. 1 ml bath.

(a) treated with physostigmine (2.5×10^{-5}M)

(b) no physostigmine

E 1 = 0.007 ml of eluate from fraction B. (Rf 0.45 to 0.53).

E 2 = 0.05 ml of eluate from fraction B. (Rf 0.45 to 0.53).

A 1 = ACh 0.08 µg. A 2 = ACh 0.7 µg. A 3 = ACh 0.5 µg.

Time, 4 min.

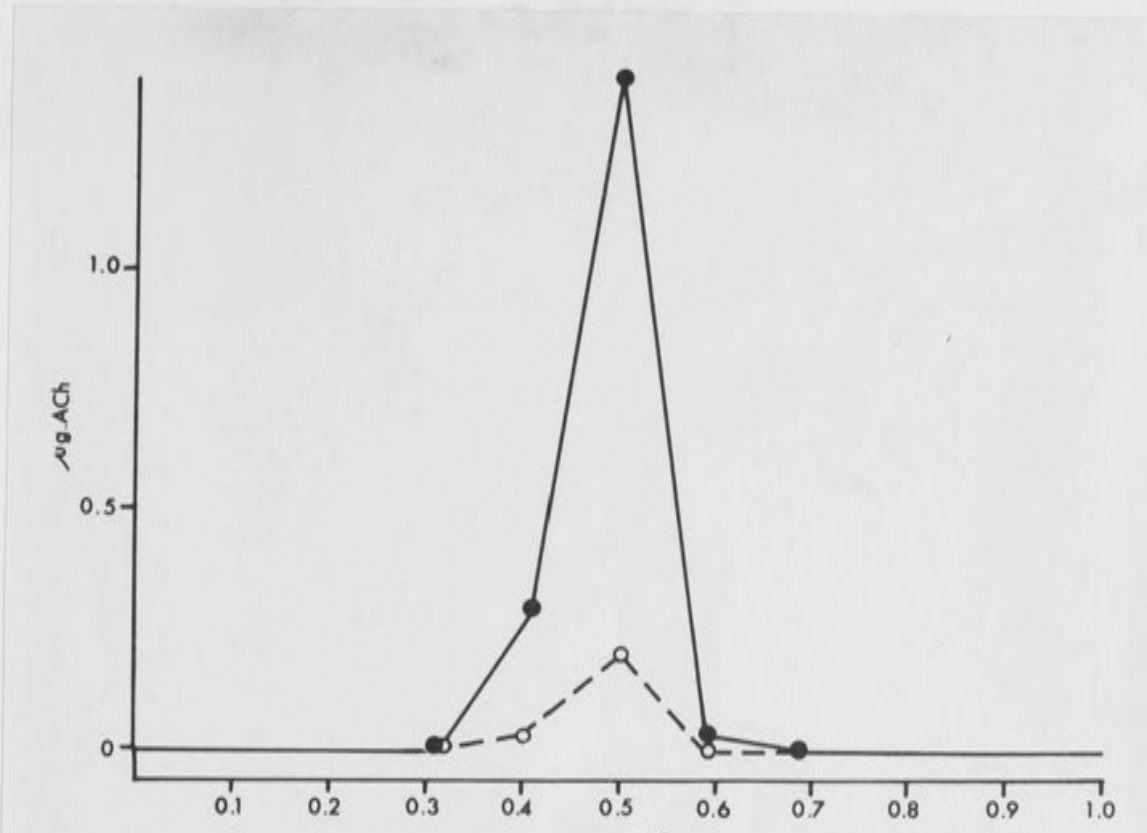

Fig. 8. Rechromatography of active components eluated from original chromatograms of fraction B. (●), peak 1 (see Fig. 4 B_1), (0) peak 3. Zero activity indicated only at the base of each peak. Assays carried out on toad atria suspended in a 1 ml bath. Ordinate is the total recovered activity expressed as µg of ACh bromide.

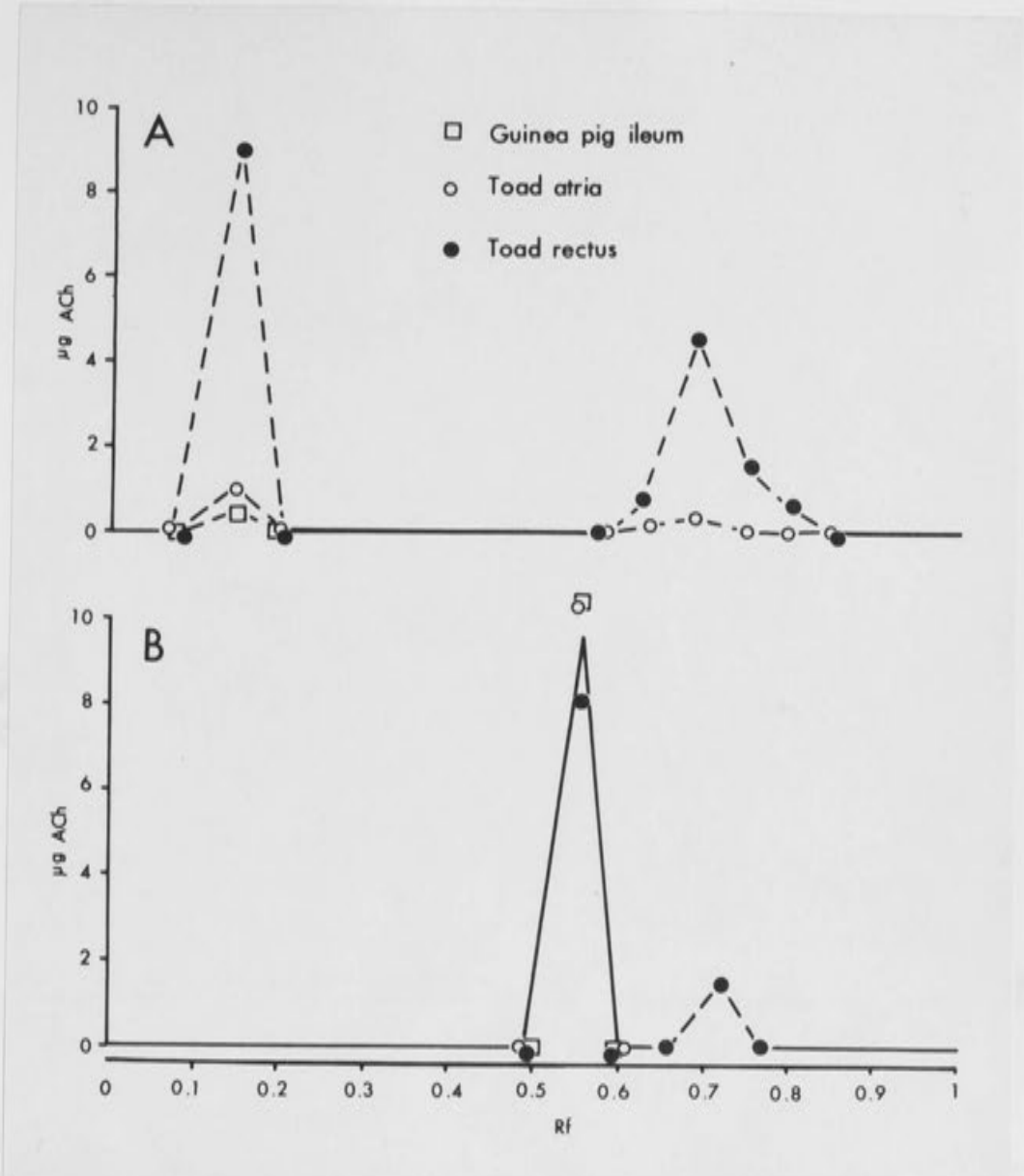

Fig. 9. Distribution of biological activity along chromatograms of (A) 'neutral' components of a brain extract, and (B) of the ACh-containing band of the electrophoretogram. Cholinomimetic activity is shown by the solid line. Non-cholinomimetic activity is shown by broken lines. Activity at Rf 0.55 is due to ACh. Activity at Rf 0.15 is due to pyridine and that at Rf 0.7 is due to an unidentified substance of non-biological origin. Electrophoresis and chromatography carried out in pyridine-containing solvents.

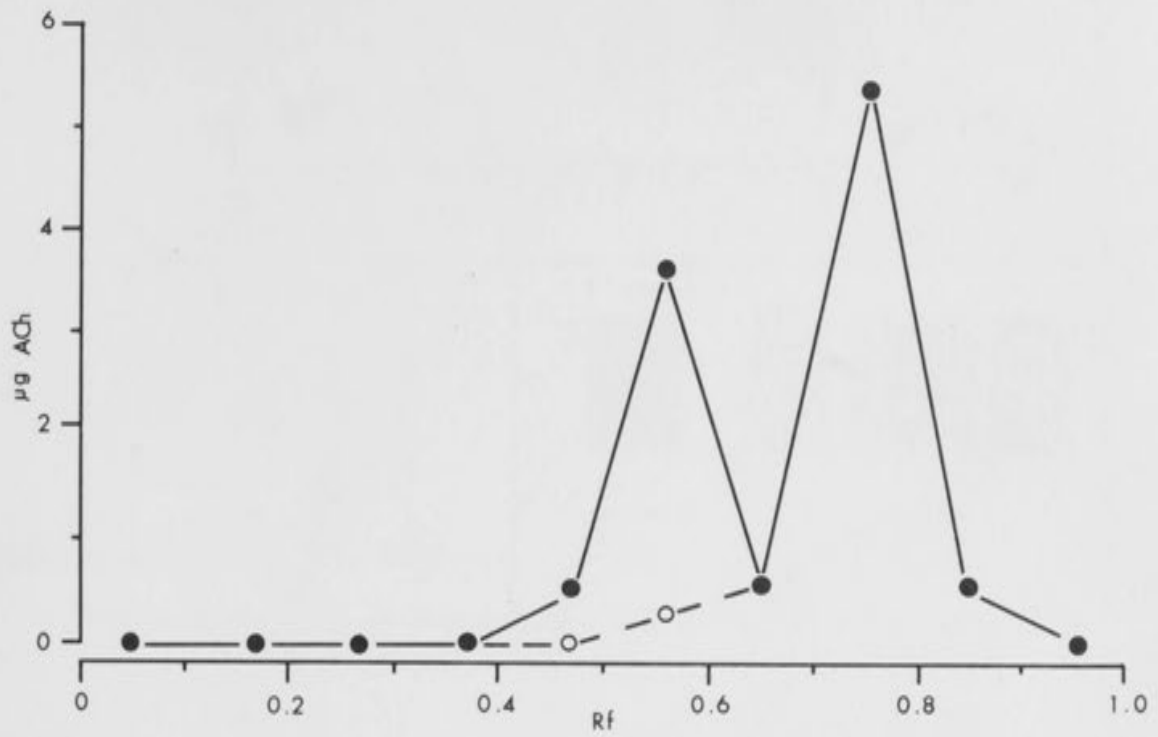

Fig. 10. Chromatography of an eluate from one whole sheet of S.S. chromatography paper, to which 3.5 µg of ACh had been added. Note the two peaks. The first, at Rf 0.55, was due to ACh and was inactivated by incubation with whole blood (open circles). The second was due to an unidentified substance eluted from the paper. Assays on toad rectus.

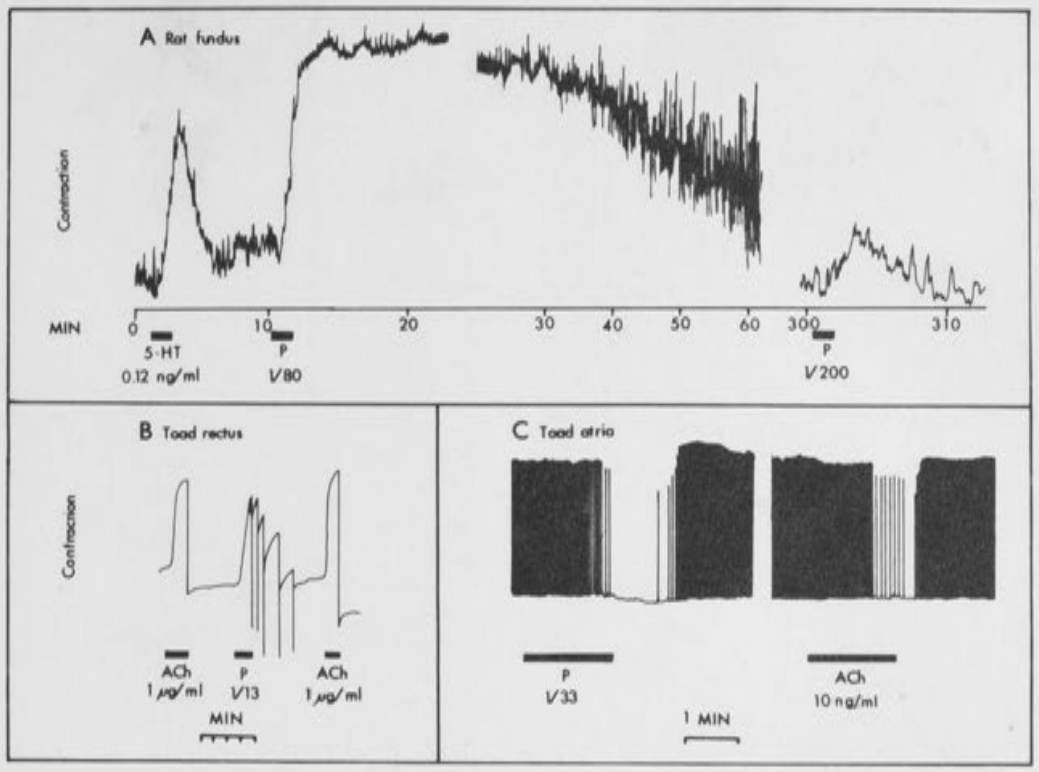

Fig. 11. Actions of an eluate from S.S. chromatography paper (P) on the isolated rat fundus, toad rectus and toad atria. The concentration is expressed as the proportion of the eluate present in 1 ml of bathing fluid. Note the prolonged action on the rat fundus and toad rectus. On the rectus the contracture persisted in spite of repeated stretching of the muscle, as indicated by the vertical lines in the record.

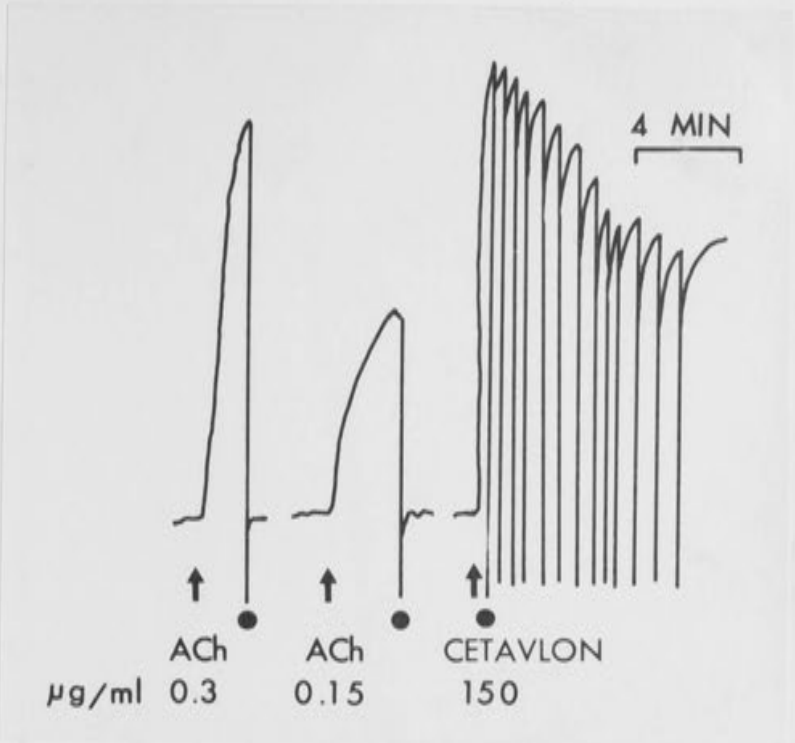

Fig. 12. Contraction of toad rectus abdominis muscle. Arbitrary scale. ACh or Cetavlon (cetyltrimethylammonium bromide) was injected into the bath at the arrows and washed out at the dots. The vertical lines projecting below the baseline were due to mechanical stretching of the muscle. Note the prolonged effect of Cetavlon.

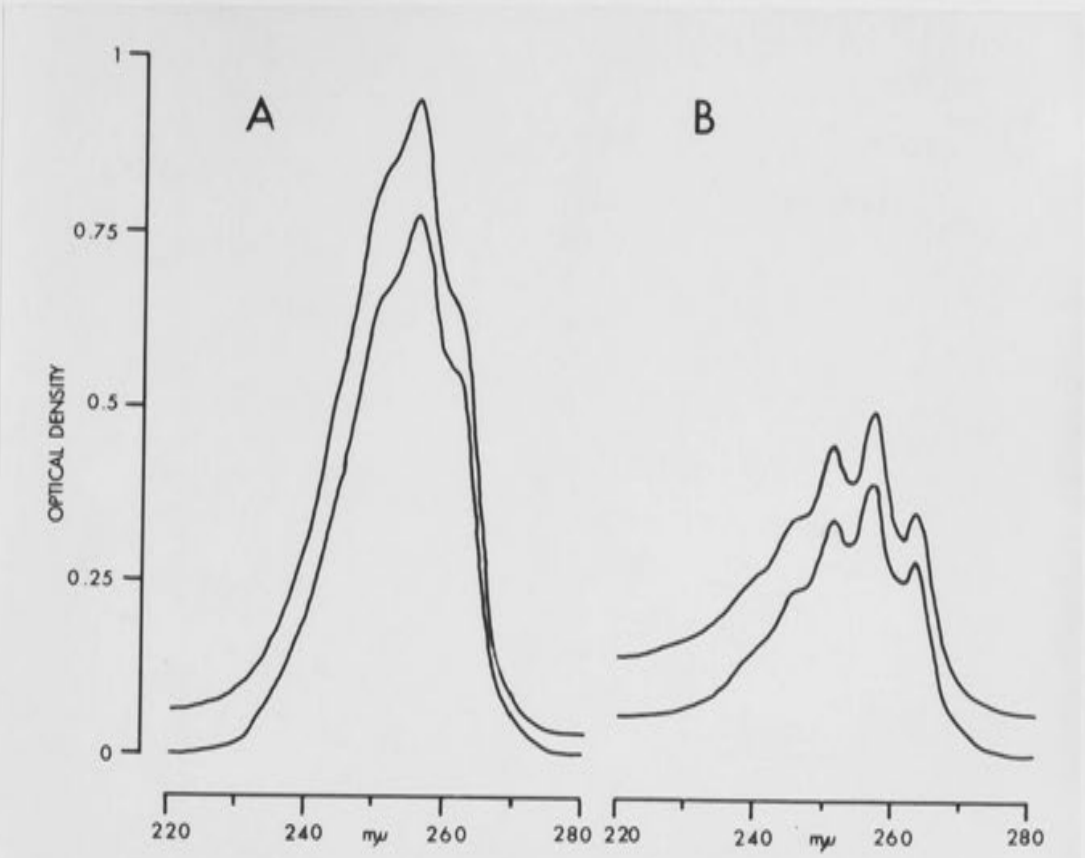

Fig. 13. Ultraviolet absorption spectra of pyridine (upper curves) and of the active low Rf (0.15) component (lower curves), from a brain extract separated in pyridine-containing buffer (see Fig. 9A).

(A), spectra in 0.05N HCl; (B), spectra in 0.1N NaOH.

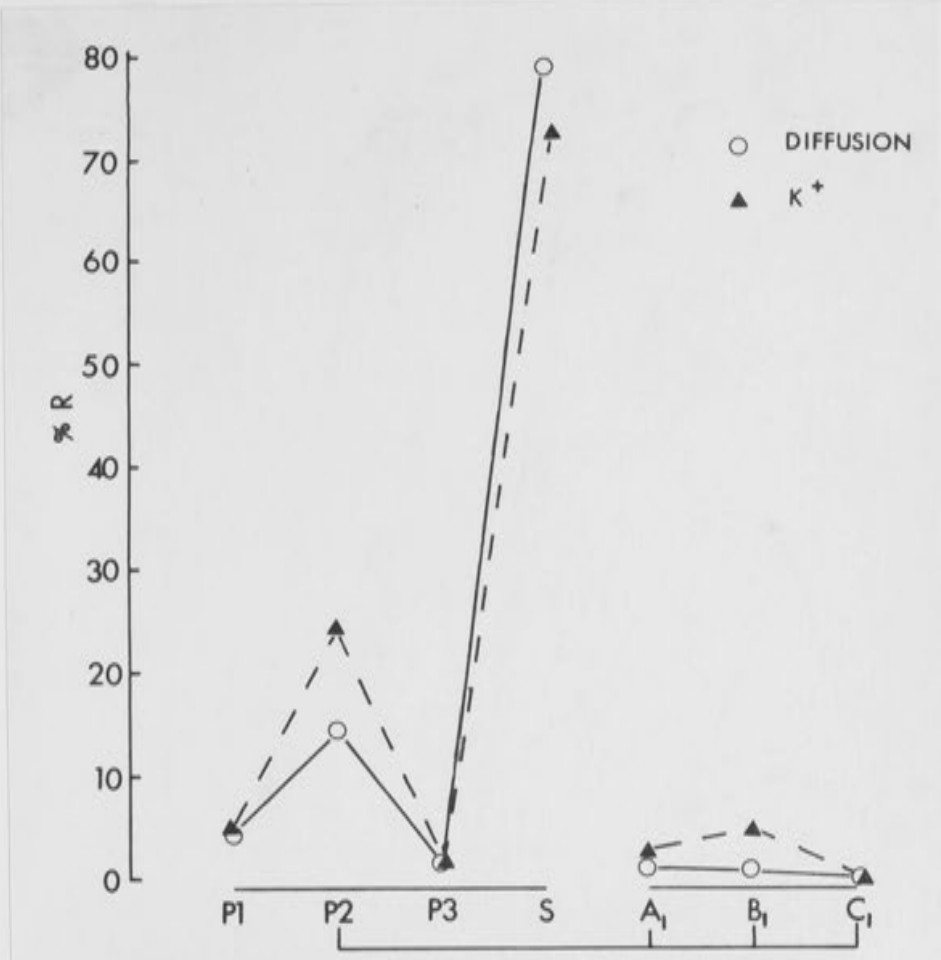

Fig. 14. Subcellular distribution in guinea pig brain homogenates of potassium (▲) and hypothetical distribution of a freely diffusible substance (○). Recovery of potassium in P1, P2, P3 and S: 66 μ equivalents/g of brain. Homogenates prepared by method (b).

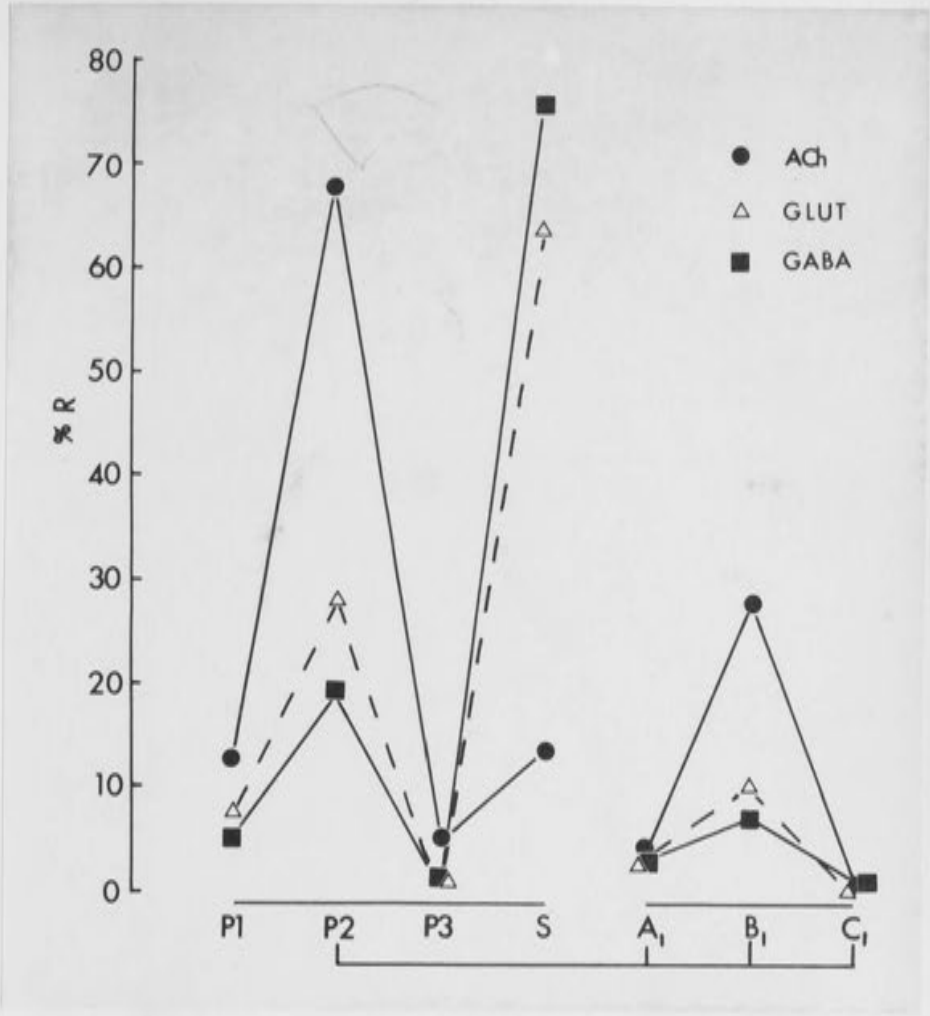

Fig. 15. Subcellular distribution in guinea pig brain homogenates of GABA (■), glutamic acid (△) and ACh (●). Recoveries in P1, P2, P3 and S: GABA, 255 μg/g of brain, glutamic acid, 793 μg/g and ACh, 0.4 μg/g. Homogenates prepared by method (b).

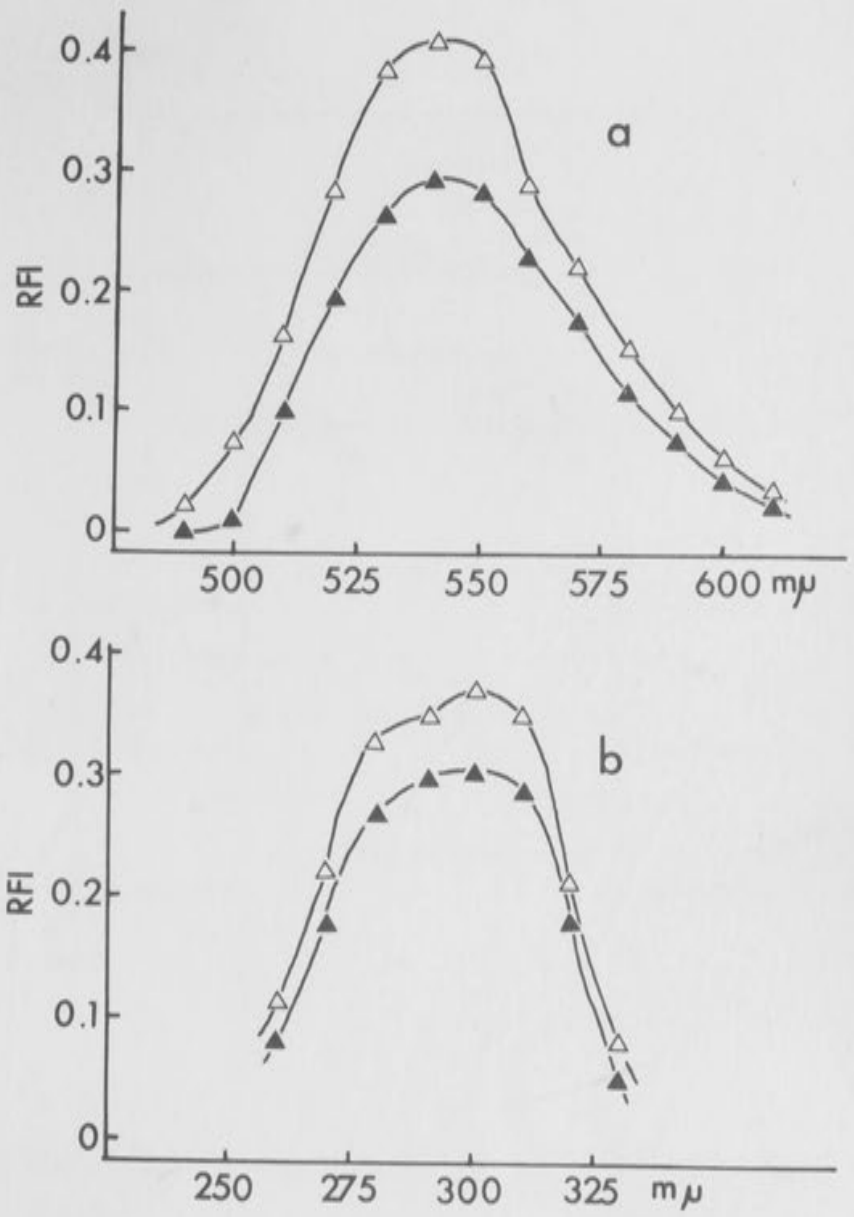

Fig. 16. A comparison of the fluorescent properties of a microsomal (P3) extract (△) and authentic 5-HT (▲) in 3N-HCl. (a) Fluorescence spectra. Activating wavelength 295 mμ. (b) Activation spectra. Fluorescence wavelength = 550 mμ. (Wavelengths uncorrected). RFI is the relative fluorescent intensity in arbitrary units.

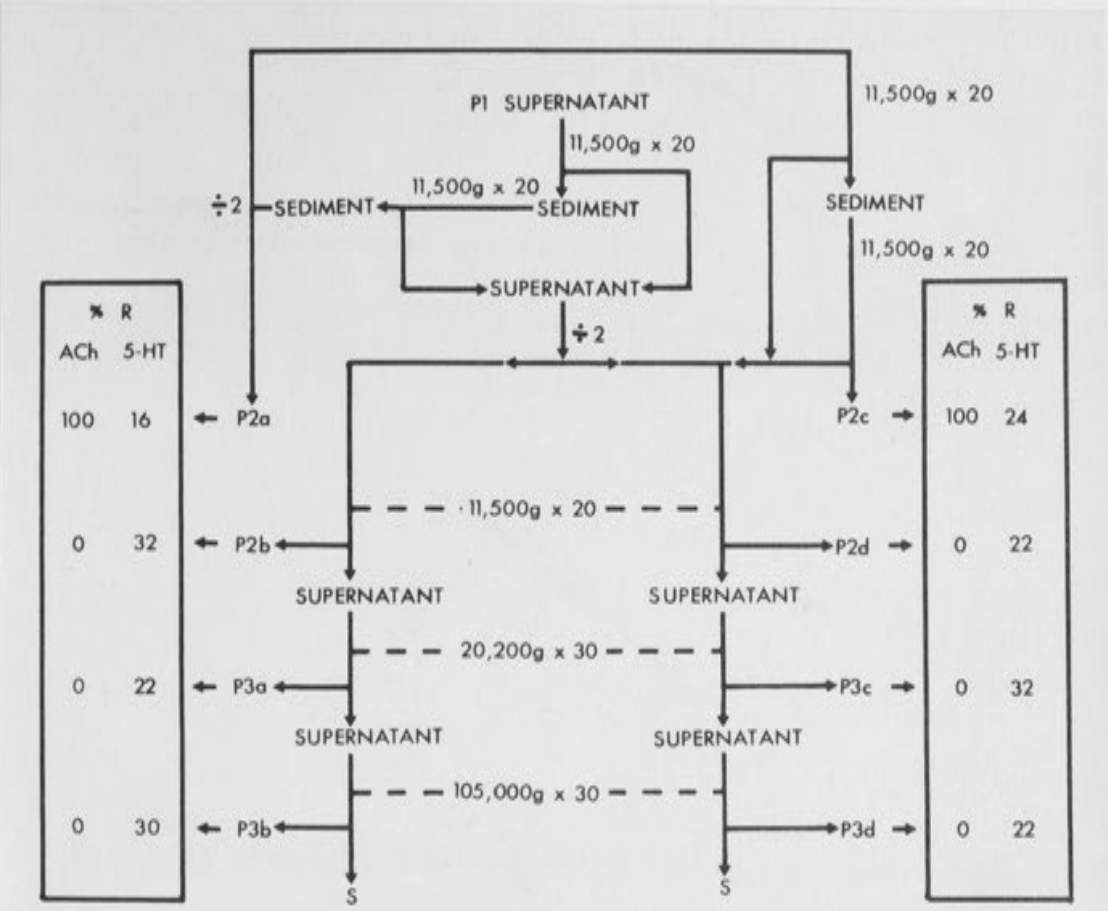

Fig. 17. Subcellular distribution of ACh and 5-HT in rat brain homogenates, which were fractionated by a modified technique. The P1 supernatant was obtained by the routine method illustrated in Tables 1 and 2. %R is the percentage of the total amount recovered in P2a, P2b, P3a and P3b or P2c, P2d, P3c and P3d. ÷ 2 shows where the sediment or supernatant were divided into two equal portions. Each figure is the average obtained in two experiments. The centrifugal fields are shown as g x min.

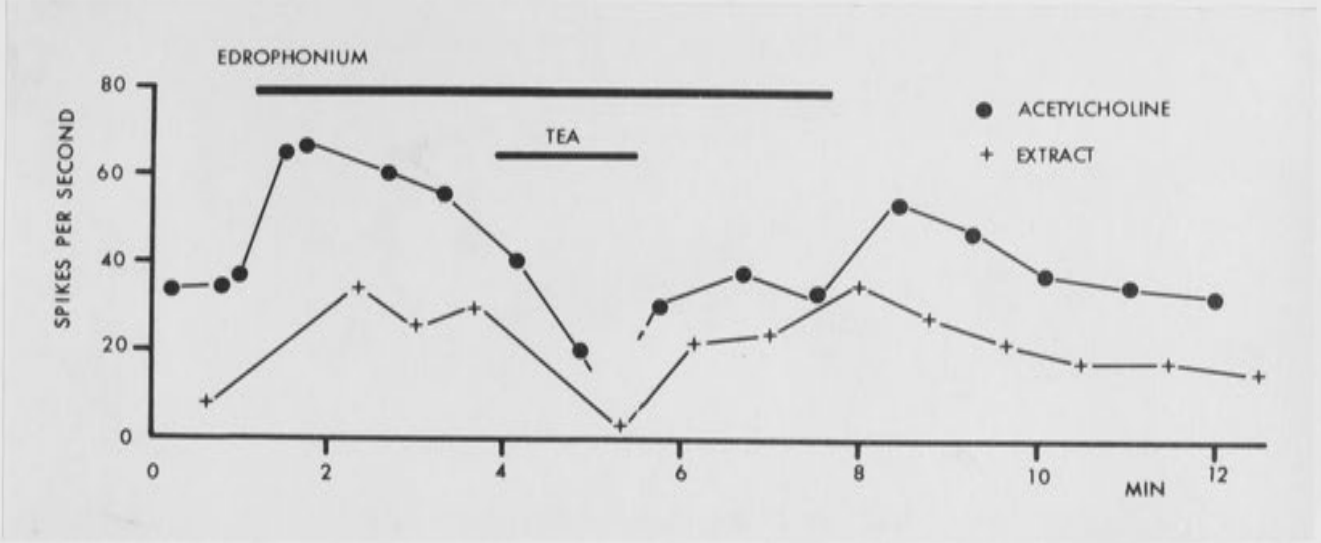

Fig. 18. Maximum firing frequencies of a single Renshaw cell (ordinate, spikes per second) in response to alternate electrophoretic ejections of authentic acetylcholine (●) and of extracted acetylcholine (+) which were made automatically for fixed periods (12 and 13 sec respectively) and at fixed intervals using currents of 20 nA. The upper horizontal bar above the plot signals the continuous ejection of edrophonium (5 nA) from a third barrel of the multiple micro-pipette and the lower horizontal bar indicates the ejection of tetraethylammonium (TEA), 10 nA, from the fourth barrel.

Abscissae, time in minutes.

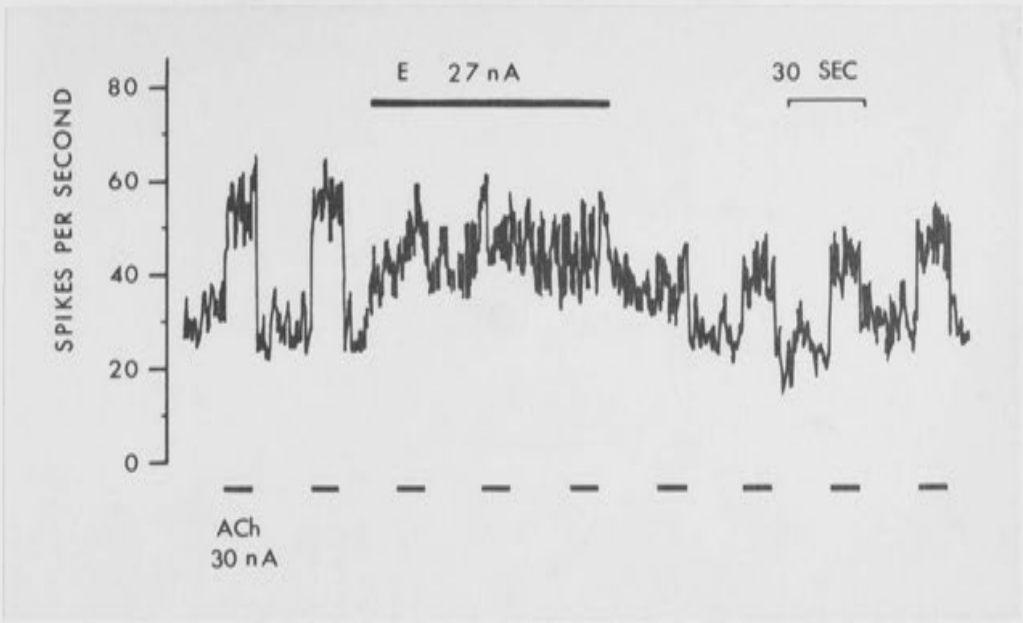

Fig. 19. Firing frequency of a Renshaw cell in response to repeated application of ACh (lower horizontal bars). E = brain extract containing ACh, applied for a period of 3 min, as indicated by the upper horizontal bar. Note: firing of cell during application of extract and concomitant reduction in response to ACh. Time 30 sec.

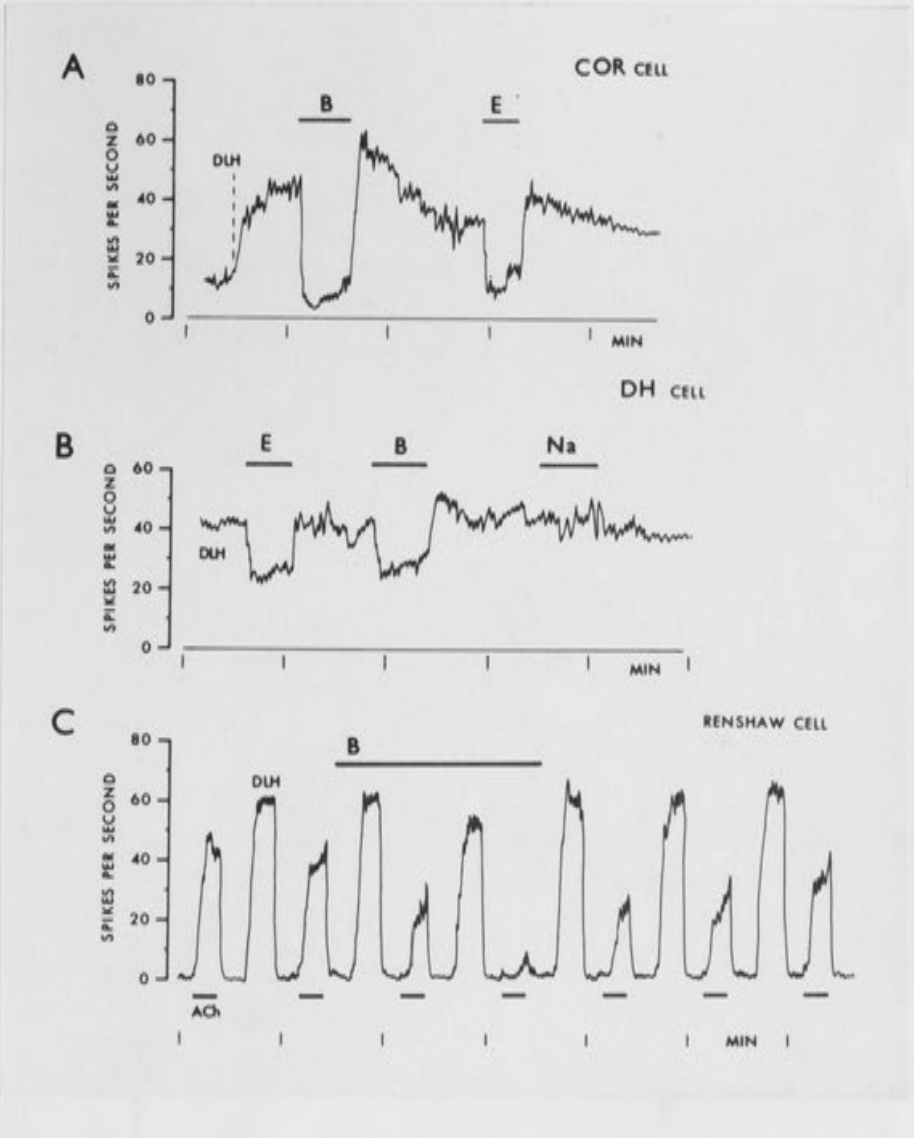

Fig. 20. Effect of electrophoretic application of cations from a purified brain extract (E) and of triethylamine (B) from a triethylamine-containing buffer, on the frequency of firing of a cortical neurone (A), an interneurone in the dorsal horn (B) and on a Renshaw cell (C): E was obtained by eluting a chromatogram with triethylamine buffer. The spontaneous firing of the cortical neurone and of the interneurone was increased by the continuous application of DL-homocysteic acid (DLH) from one barrel of the micro-electrode. In C, the effect of a continuous application of triethylamine on the firing induced by alternate ejections of ACh and of DLH was observed. Time - minutes.

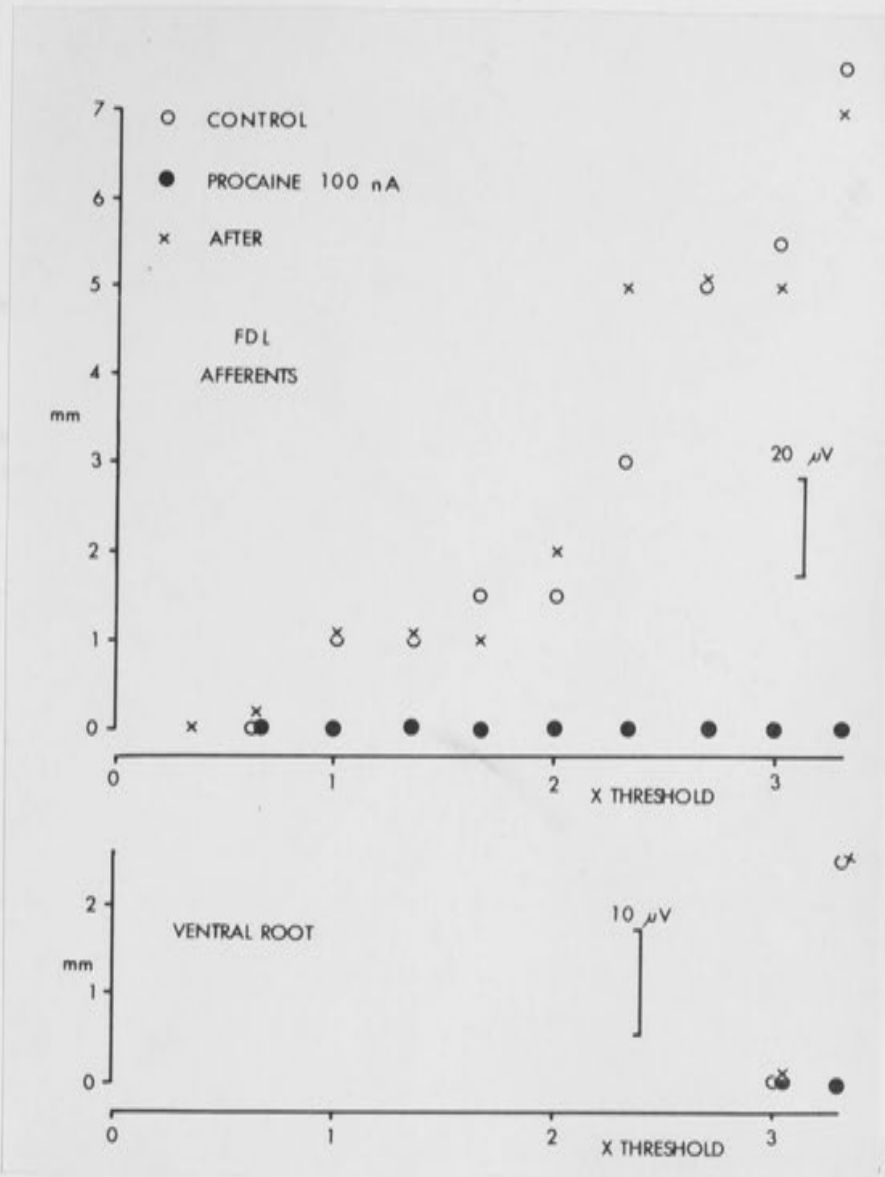

Fig. 21. Action of procaine on the excitability of primary afferent fibres of the flexor digitorum longus (FDL) nerve and of the motoneuronal somas in the anterior horn of the spinal cord.

Ordinate: size of the response as measured from the filmed record (mm).

Abscissae: stimulus strength expressed in terms of the threshold stimulus (9V) for the primary afferent fibres.

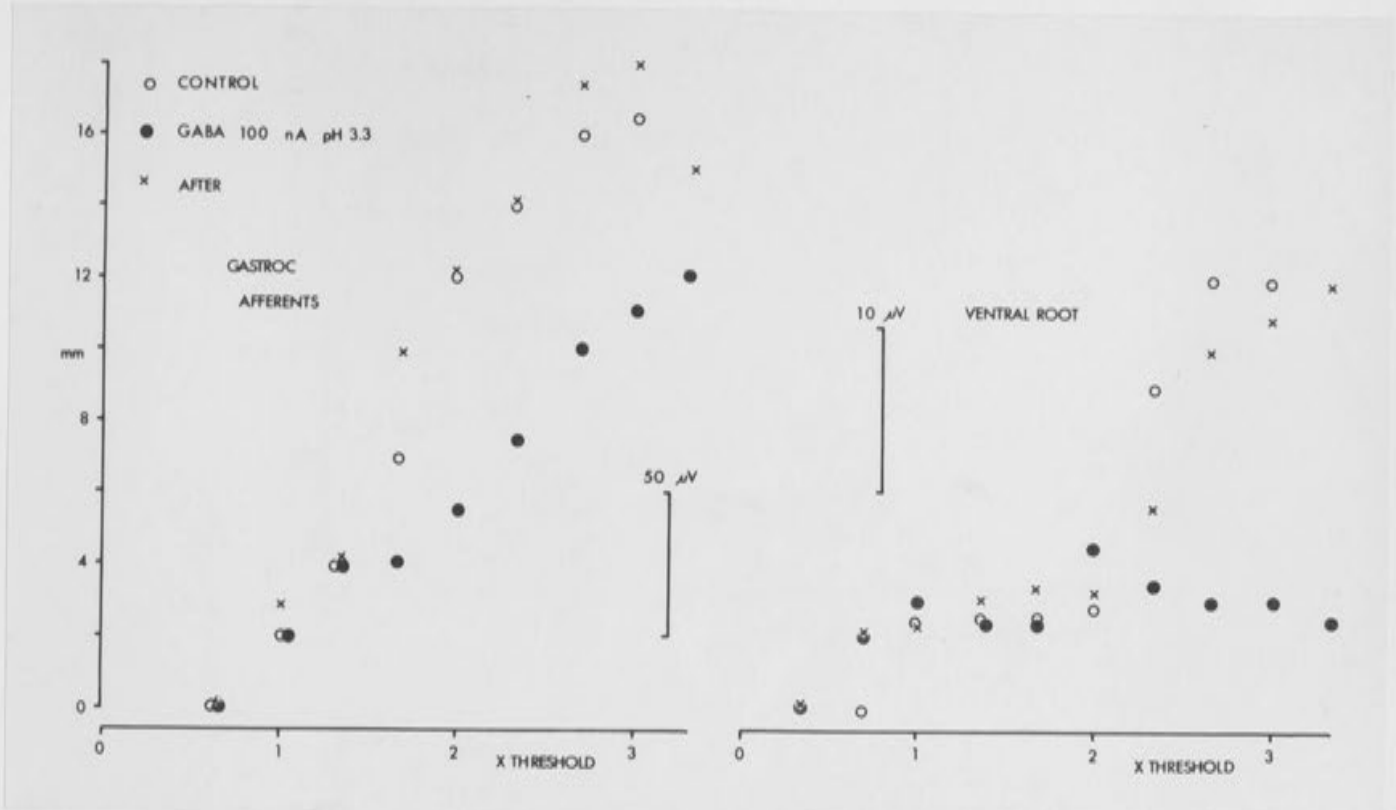

Fig. 22. Action of GABA (pH 3.3) on the excitability of gastrocnemius group Ia fibres and motoneuronal somas in the anterior horn of the spinal cord.

Ordinates: size of the response as measured from the filmed records (mm).

Abscissae: stimulus strength expressed in terms of the threshold stimulus (18V) for the primary afferent fibres.

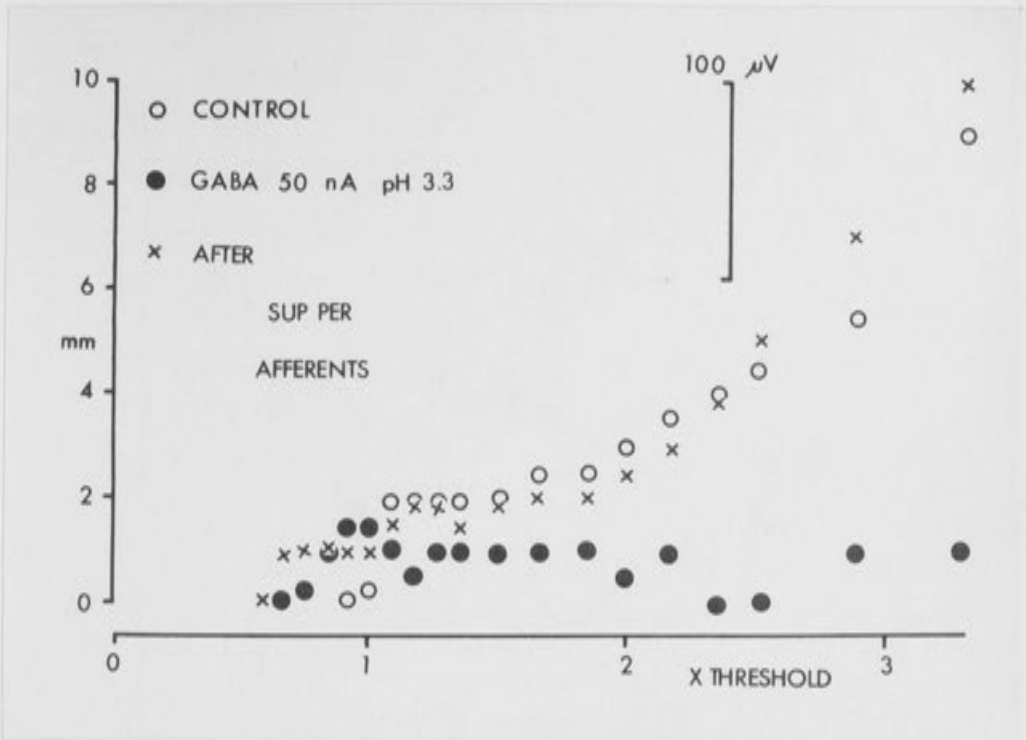

Fig. 23. Action of GABA (pH 3.3) on the excitability of primary afferent cutaneous fibres in the dorsal horn of the spinal cord.

Ordinates: size of the response as measured from the filmed records (mm).

Abscissae: stimulus strength, expressed in terms of the threshold stimulus (IIV) for the primary afferent fibres.

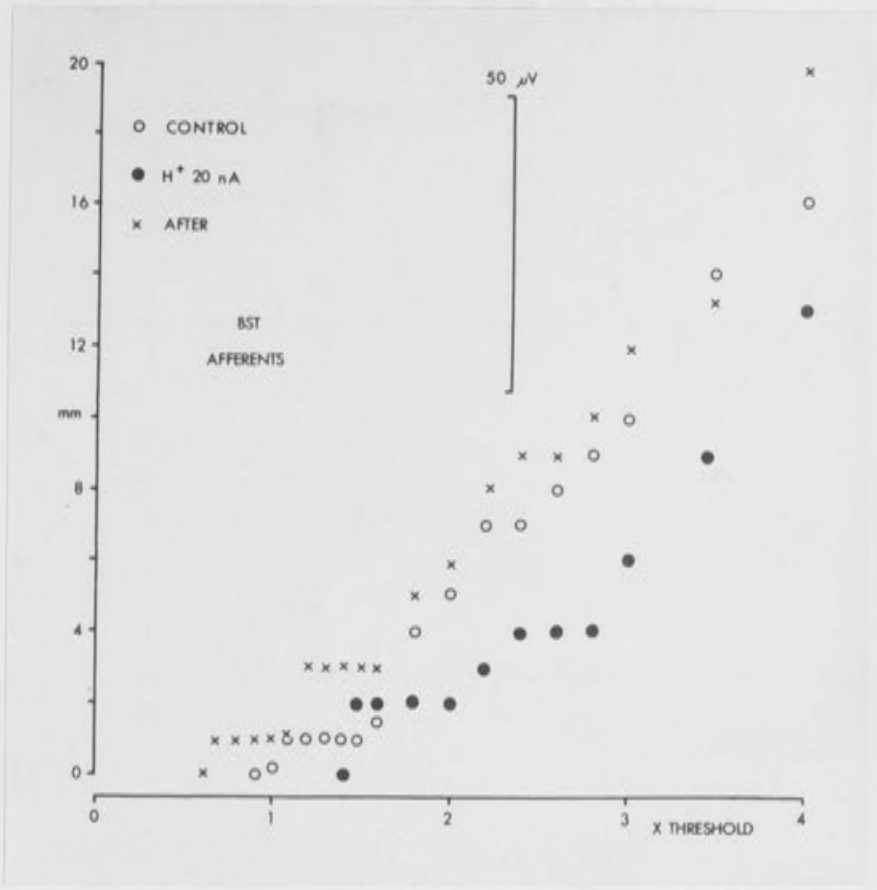

Fig. 24. Action of hydrogen ions on the excitability of primary afferent fibres of the biceps semitendinosus nerve (BST) in the anterior horn of the spinal cord.

Ordinates: size of the response as measured from the filmed record (mm).

Abscissae: stimulus strength expressed in terms of the threshold stimulus (14V) for the primary afferent fibres.

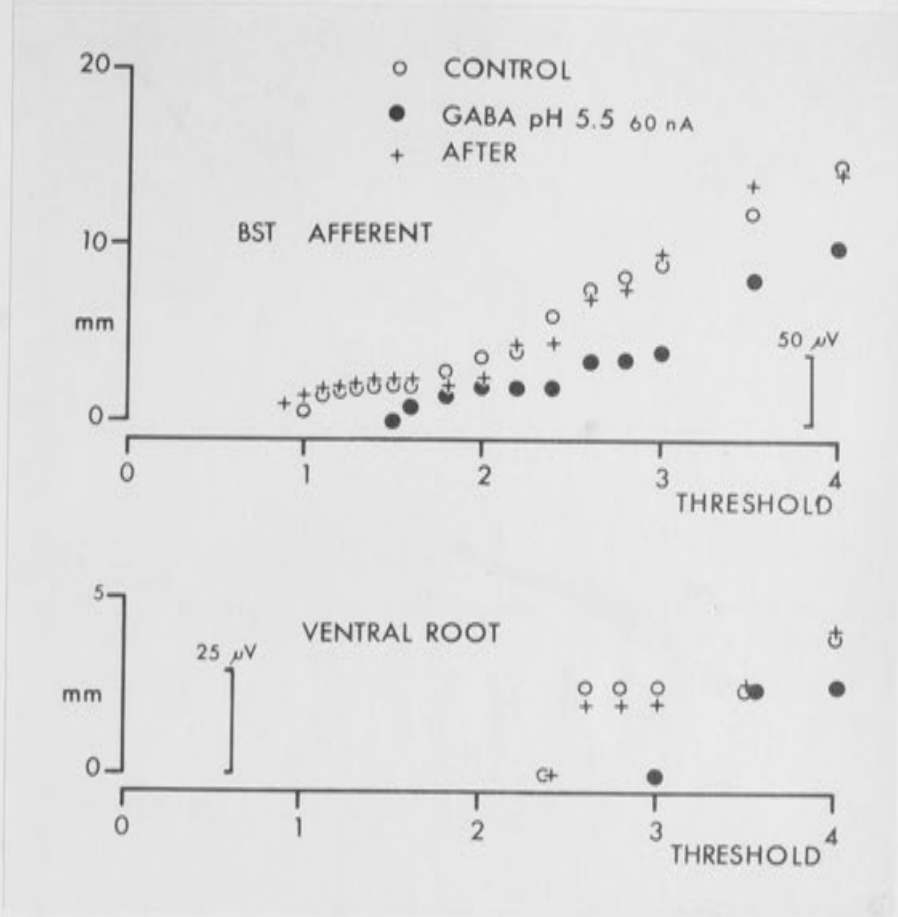

Fig. 25. Action of GABA (pH 5.5) on the excitability of primary afferent fibres of the biceps semitendinosus (BST) nerve and of the motoneuronal somas in the anterior horn of the spinal cord.

Ordinates: size of the response as measured from the filmed record (mm).

Abscissae: stimulus strength expressed in terms of the threshold stimulus (6.4V) for the primary afferent fibres.

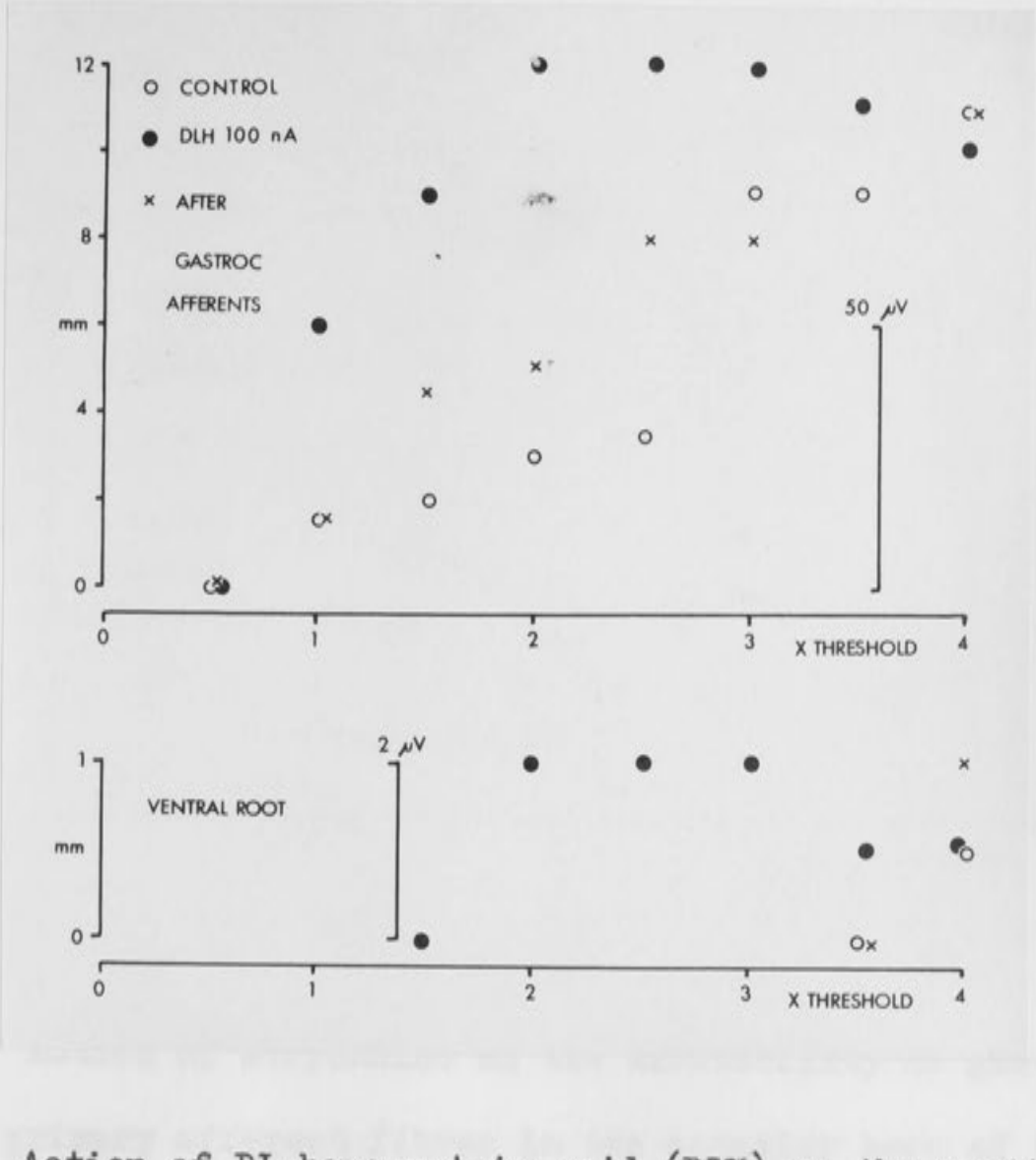

Fig. 26. Action of DL-homocysteic acid (DLH) on the excitability of gastrocnemius group Ia primary afferent fibres and of the motoneuronal somas in the anterior horn of the spinal cord.

Ordinates: size of the response as measured from the filmed record (mm).

Abscissae: stimulus strength expressed in terms of the threshold stimulus (10V) for the primary afferent fibres.

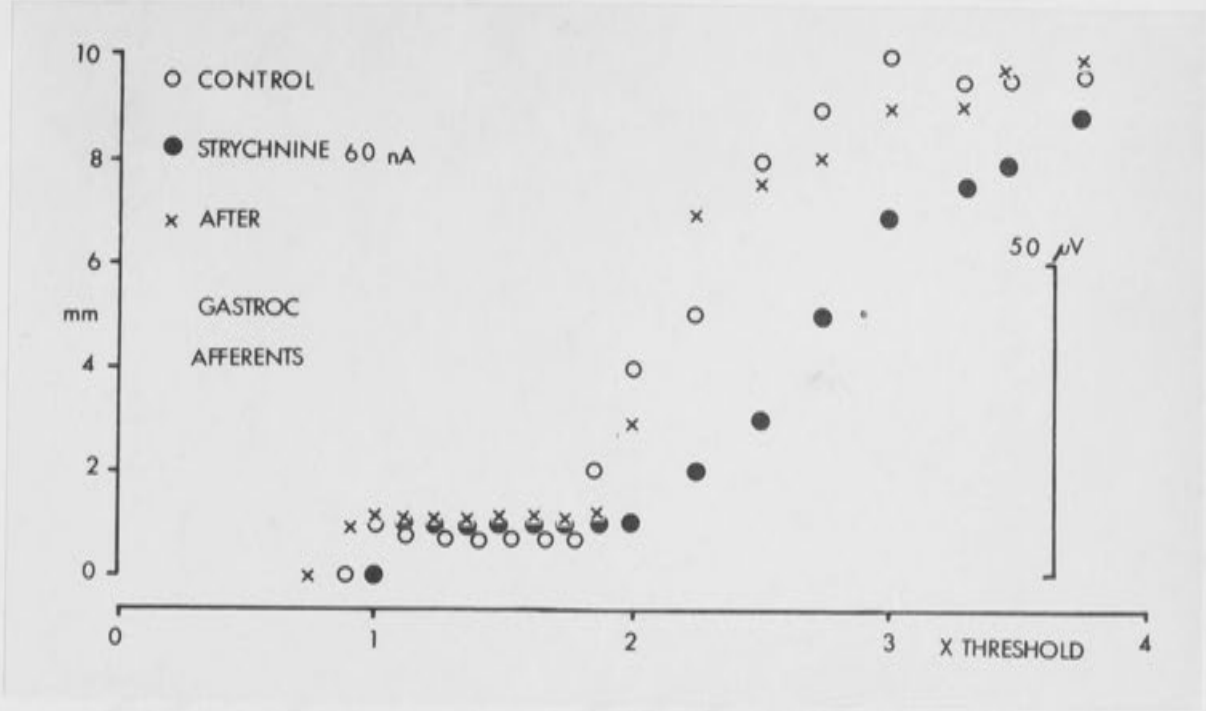

Fig. 27. Action of strychnine on the excitability of gastrocnemius group Ia primary afferent fibres in the anterior horn of the spinal cord.

Ordinates: size of the response as measured from the filmed record (mm)

Abscissae: stimulus strength expressed in terms of the threshold stimulus (llV) for the primary afferent fibres.

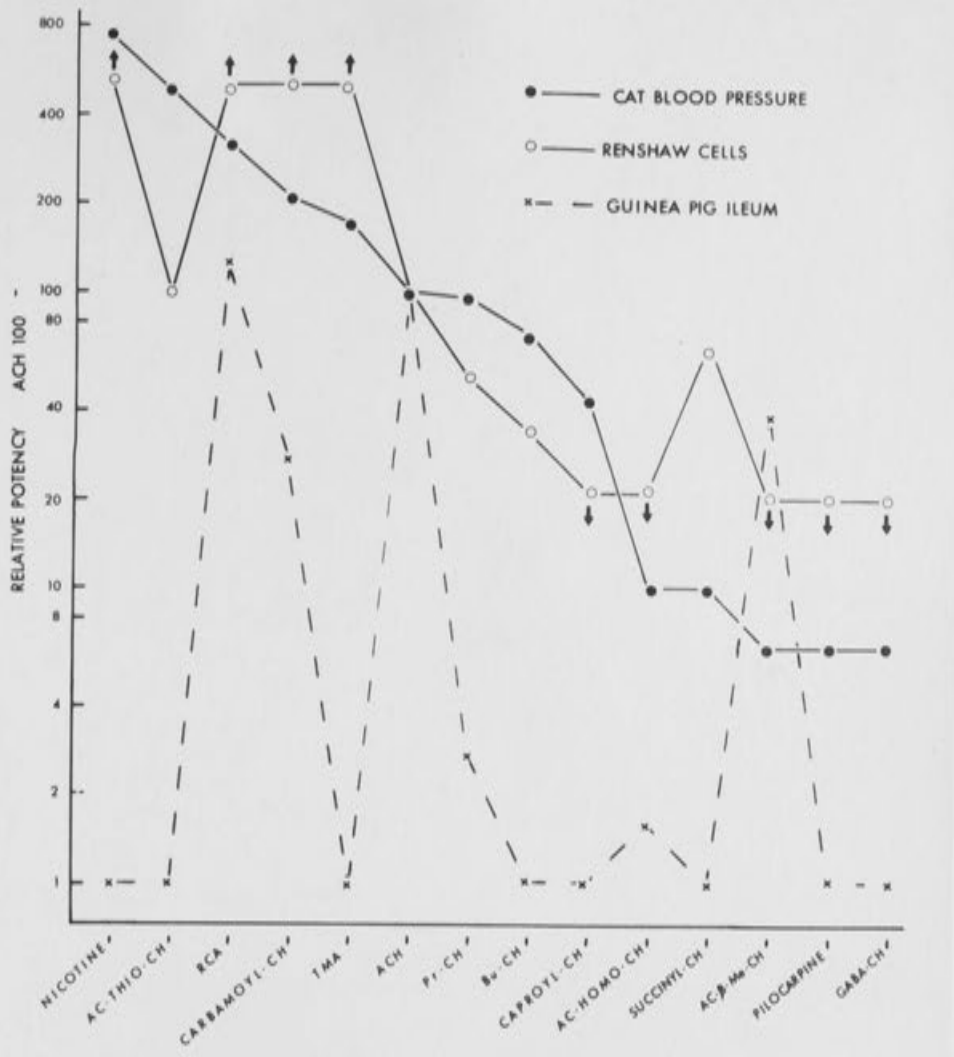

Fig. 28. Potency of cholinomimetic substances relative to that of ACh (= 100) plotted on the ordinate (log scale), determined on the blood pressure of the atropinized cat (closed circles), on Renshaw cells (open circles) and on the guinea pig ileum (crosses). The compounds are arranged along the abscissa in descending order of activity determined on the blood pressure. Arrows pointing up indicate that the substances were more than four times as potent as ACh on Renshaw cells and arrows pointing down indicate that they were less than one-fifth as potent. The compounds listed along the abscissa are; nicotine, acetylthiocholine (AC-THIO-CH), γ-propiobetaine methyl ester (RCA), carbamoylcholine (CARBAMOYL-CH), tetramethylammonium (TMA), ACh, propionylcholine (Pr-CH), n-butyrylcholine (Bu-CH), caproylcholine (CAPROYL-CH), acetylhomocholine (AC-HOMO-CH), succinylcholine (SUCCINYL-CH), acetyl-β-methylcholine (AC-β-Me-CH), pilocarpine and γ-amino-n-butyrylcholine (GABA-CH).

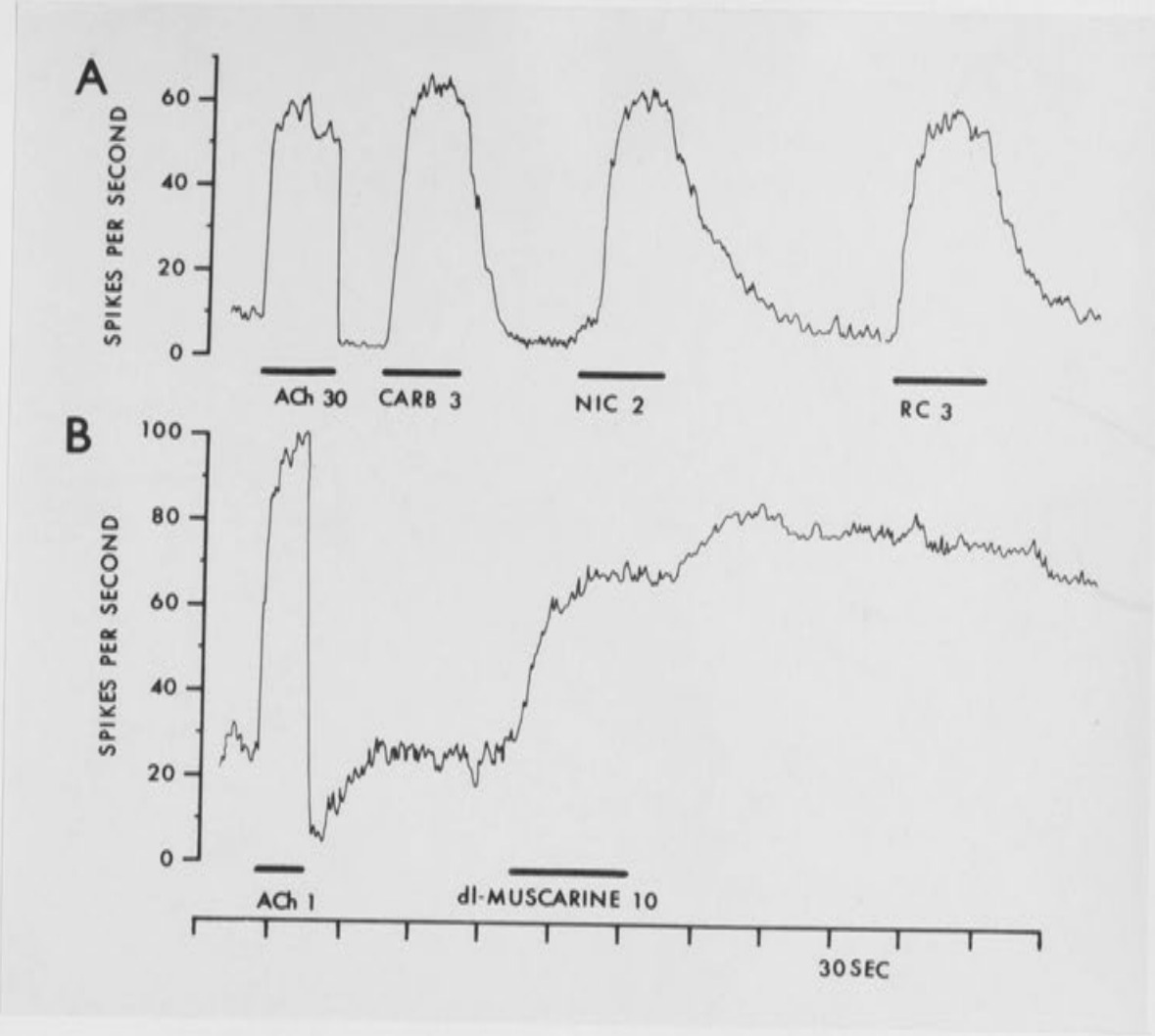

Fig. 29. Firing frequency (ordinate) of a Renshaw cell plotted against time (abscissa). A, response to ACh, 30 nA; carbamylcholine 3 nA (CARB); nicotine, 2 nA (NIC 2); γ-propiobetaine methyl ester, 3 nA (RC 3): B, responses to ACh, 1 nA and dl-muscarine, 10 nA, in another Renshaw cell: Time 30 sec.

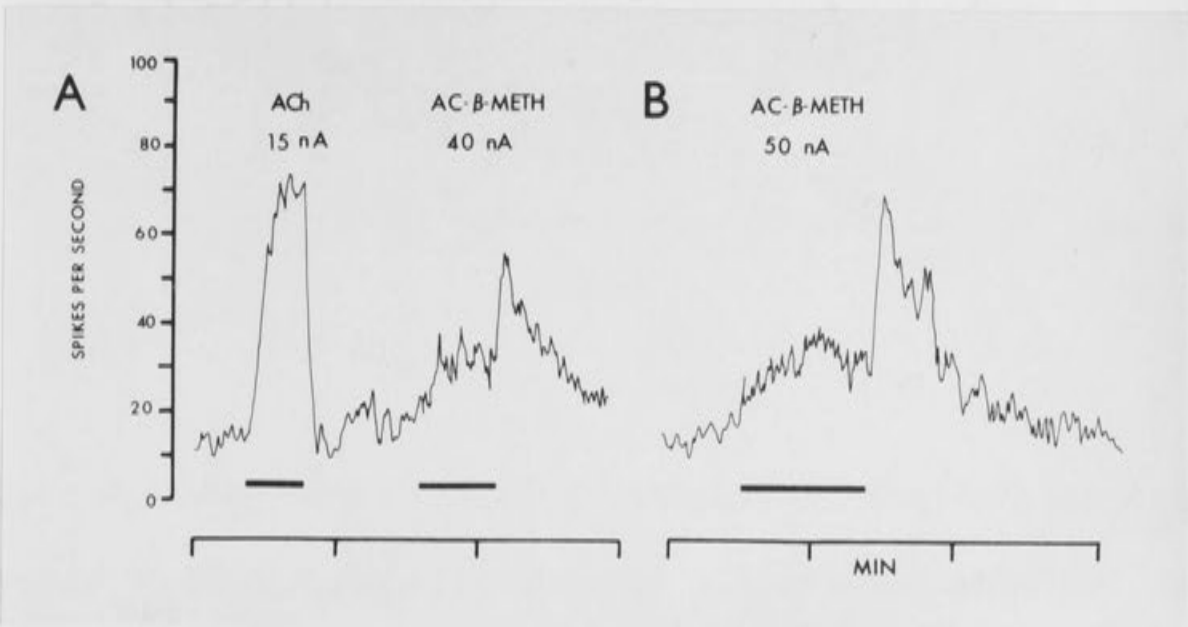

Fig. 30. Frequency of firing (ordinate) of a Renshaw cell. Effects produced by the electrophoretic ejection of ACh and acetyl-β-methylcholine (AC-β-METH) during the periods indicated by the lower horizontal bars. Time, min.

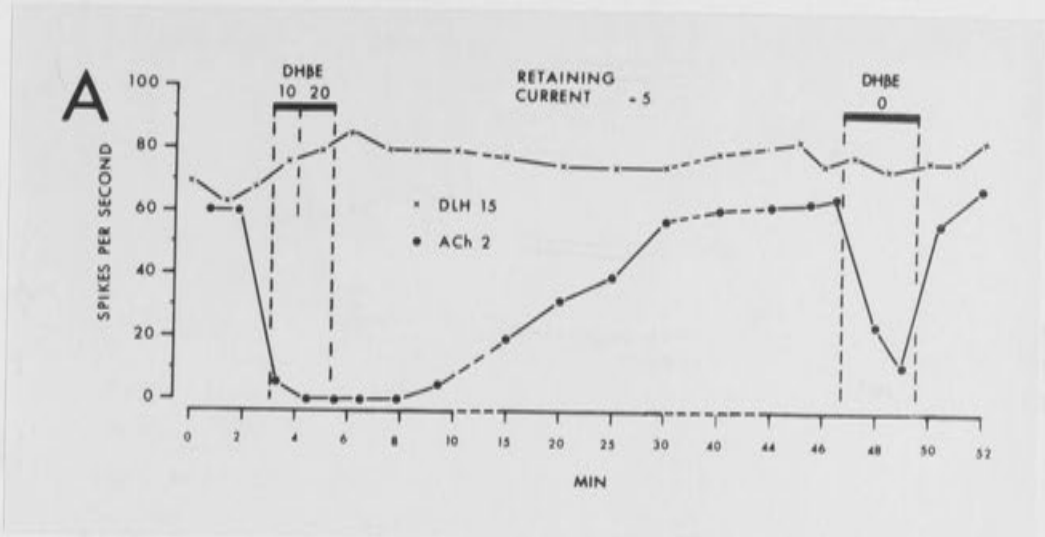

Fig. 31. A, Frequency of firing (ordinate) of a Renshaw cell in response to alternate ejections of ACh (closed circles) and DLH (crosses). Dihydro-β-erythroidine (DHβE) was ejected with currents of 10 and 20 nA (left hand side of figure), or was allowed to diffuse passively from the electrode by removing the retaining current(DHβE, 0).

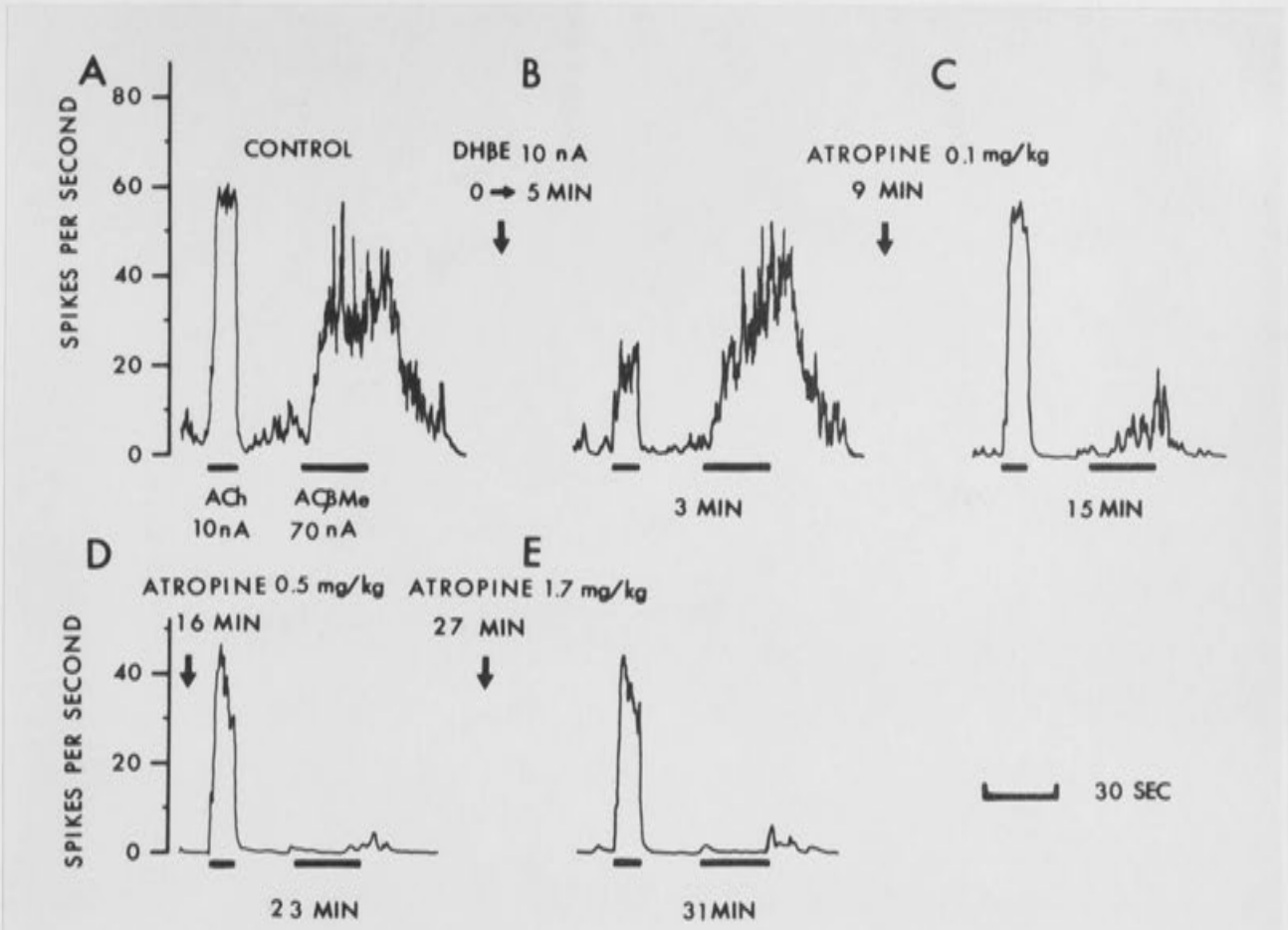

Fig. 32. Frequency of firing (ordinate) of a Renshaw cell. Responses to alternate applications of ACh or acetyl-β-methylcholine during the periods indicated by the horizontal bars. A, control responses; B, 3 min after the beginning of an ejection of dihydro-β-erythroidine (DHβE); C, the DHβE ejection ceased just after record B was obtained, atropine was then injected intravenously and 6 min later the records in C were obtained; D, 7 min after the injection of an additional 0.5 mg/kg of atropine; E, 4 min after an additional 1.7 mg/kg of atropine. Time, 30 sec.

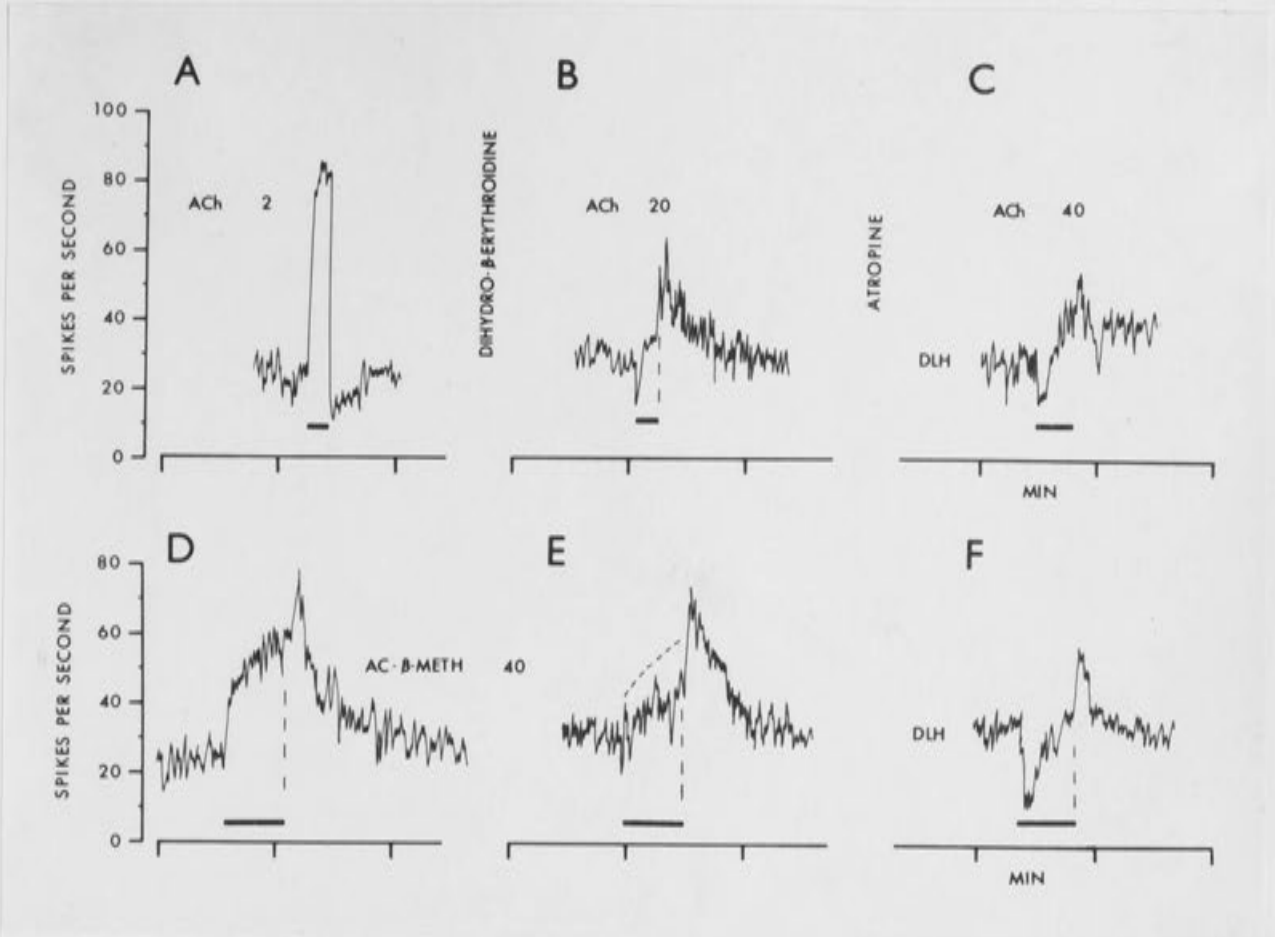

Fig. 33. Frequency of firing (ordinate) of a Renshaw cell plotted against time in min (abscissa). A, B and C, ACh ejected with currents of 2, 20 and 40 nA respectively; D, E and F, Acetyl-β-methylcholine ejected with a current of 40 nA; A and D control responses; B and E, records obtained approximately 7 min after the begining of an ejection of DHβE (20 nA) from a 0.1 M solution; atropine sulphate (0.7 mg/kg) was injected intravenously between records B and E and records C and F; this reduced the background rate of firing to zero; C and F, the background rate of firing was increased to the original level by a continuous ejection of DLH (8 nA).
N.B. DHβE was ejected continously in records B, E, C and F.

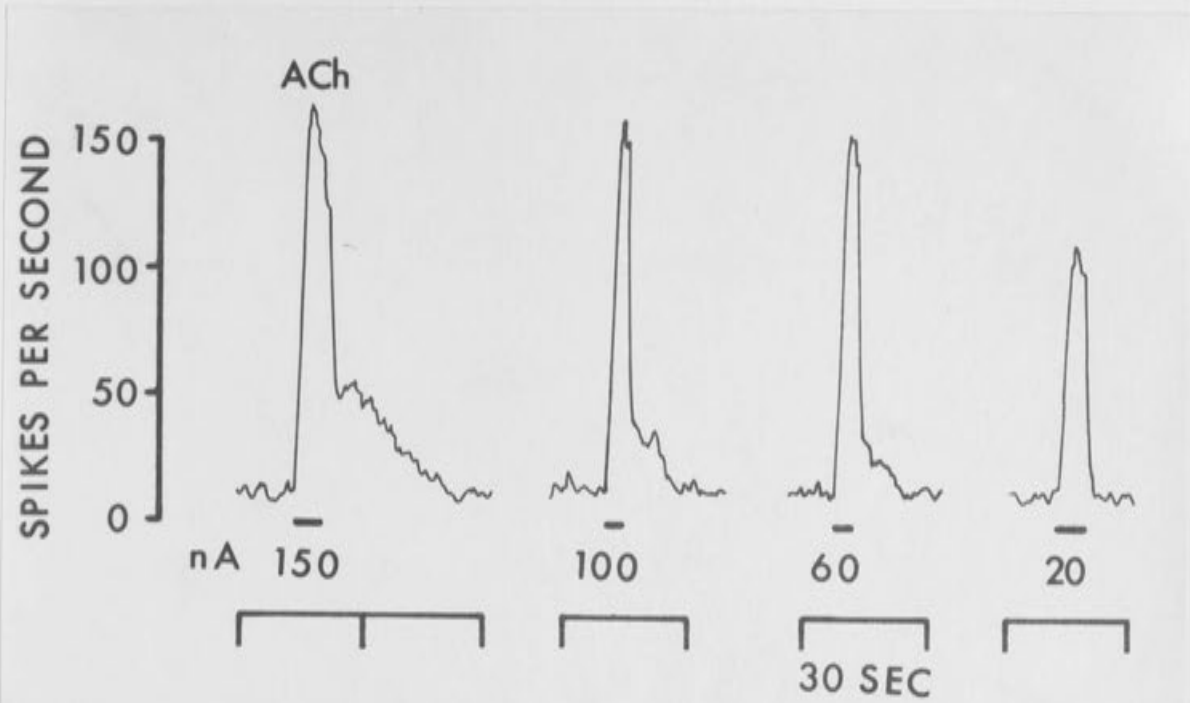

Fig. 34. Frequency of firing (ordinate) of a Renshaw cell in response to brief current pulses of ACh. Time 30 sec. Note the biphasic recovery after large currents were passed.

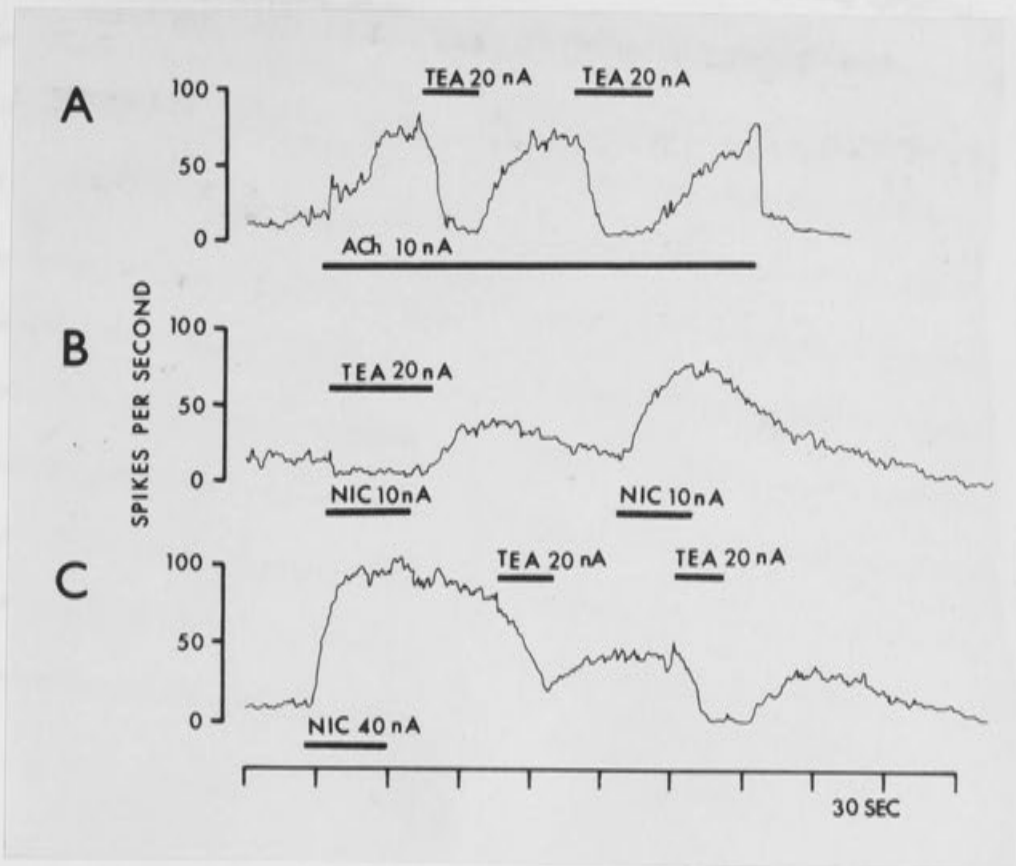

Fig. 35. Frequency of firing (ordinate) of a Renshaw cell. A) effect of brief applications of tetraethylammonium (TEA, upper horizontal bars) on the firing produced by the continuous ejection of ACh (lower horizontal bar); B), effect obtained when nicotine (NIC, lower horizontal bar) and tetraethylammonium (TEA, upper horizontal bar) were applied simultaneously; C) firing was produced by a brief ejection of nicotine (NIC, lower horizontal bar) and tetraethylammonium was ejected for brief periods during the recovery (TEA, upper horizontal bars). Time, 30 sec.

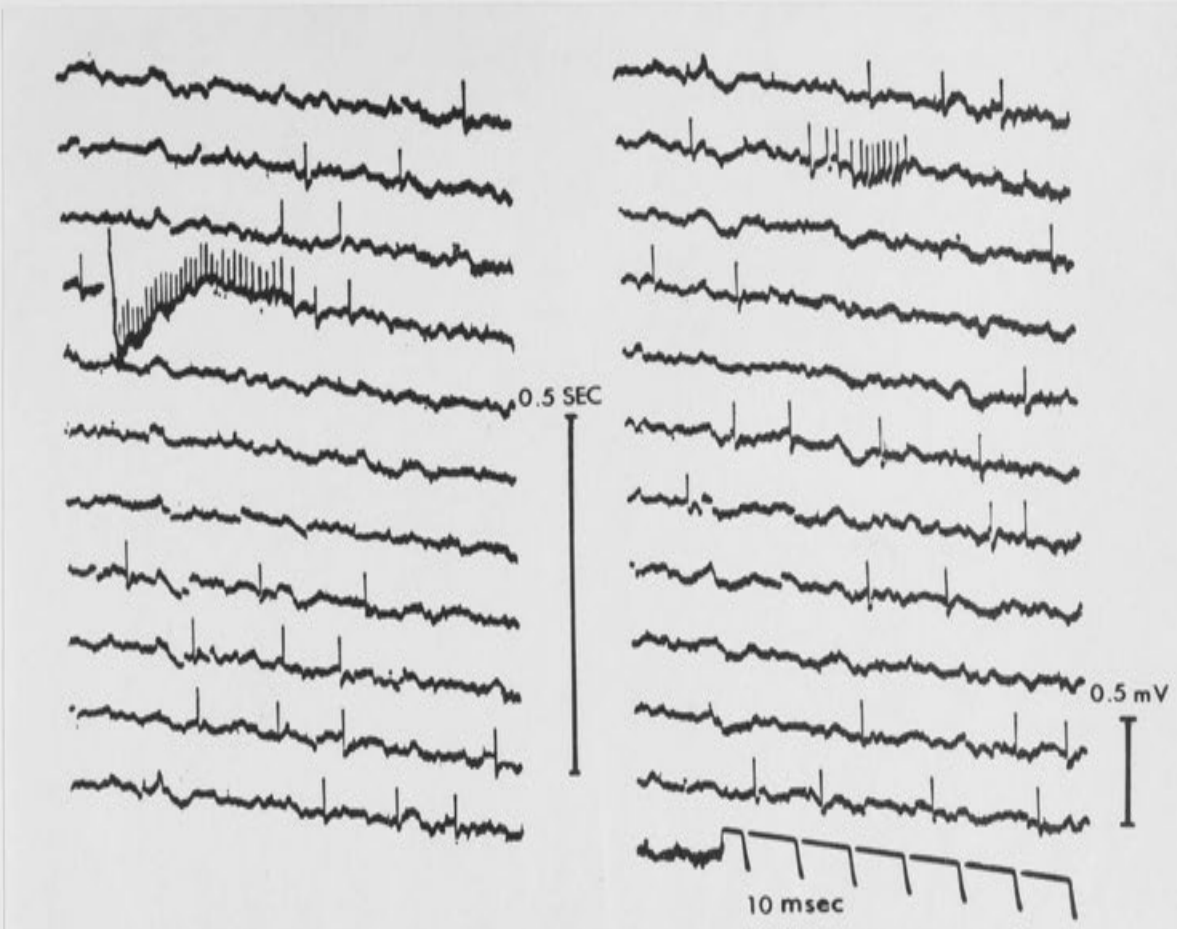

Fig. 36. Extracellular spike potentials of a Renshaw cell recorded on moving film before during and after a single supra-maximal shock was applied to the ventral root. Sweep duration 80 msec, 12 sweeps per sec. Calibrations; 0.5 sec for film speed, 0.5 mV and 10 msec.

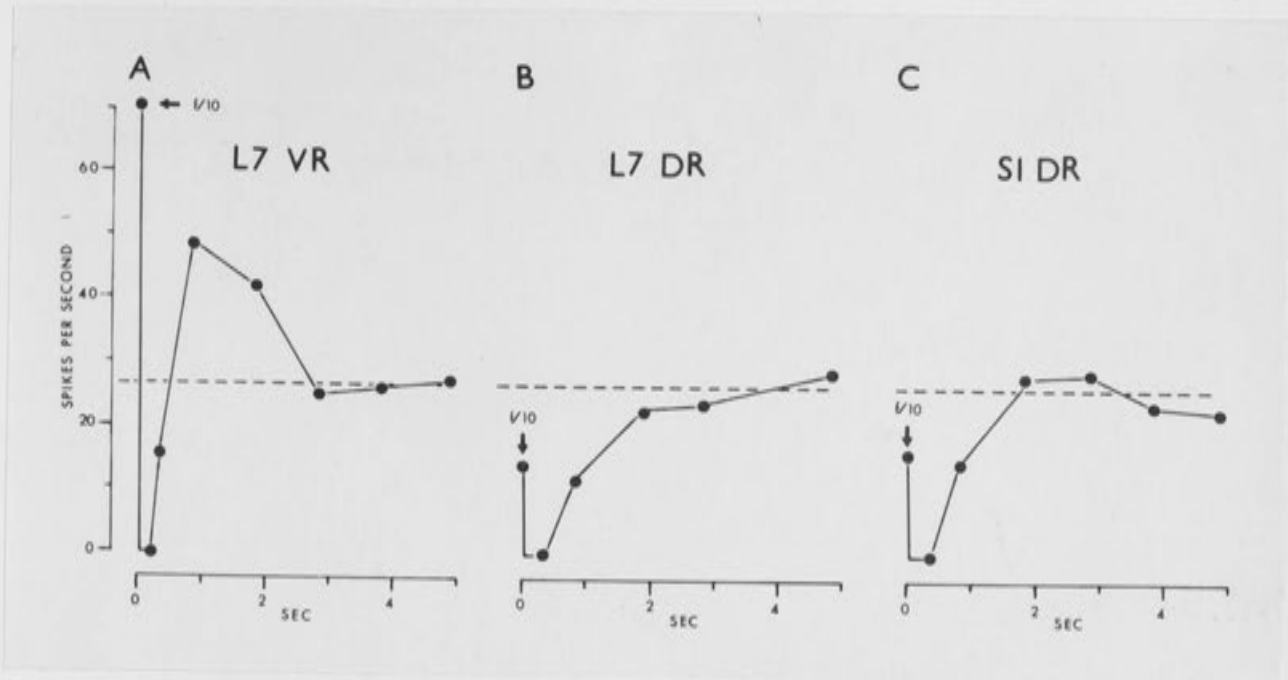

Fig. 37. Frequency of firing (ordinate) of a Renshaw cell, plotted against time (abscissae). Note that the average frequencies of the initial responses have been divided by 10 (indicated by f/10). Dotted lines represent the average spontaneous rate of firing. Response following A) a single shock to the ventral root, B) a single shock to the L7 dorsal root and C) a single shock to the S1 dorsal root. Note the late response following ventral root stimulation, which was absent when dorsal roots were stimulated.

Time, sec after stimulus. Each point is the average for three separate volleys.

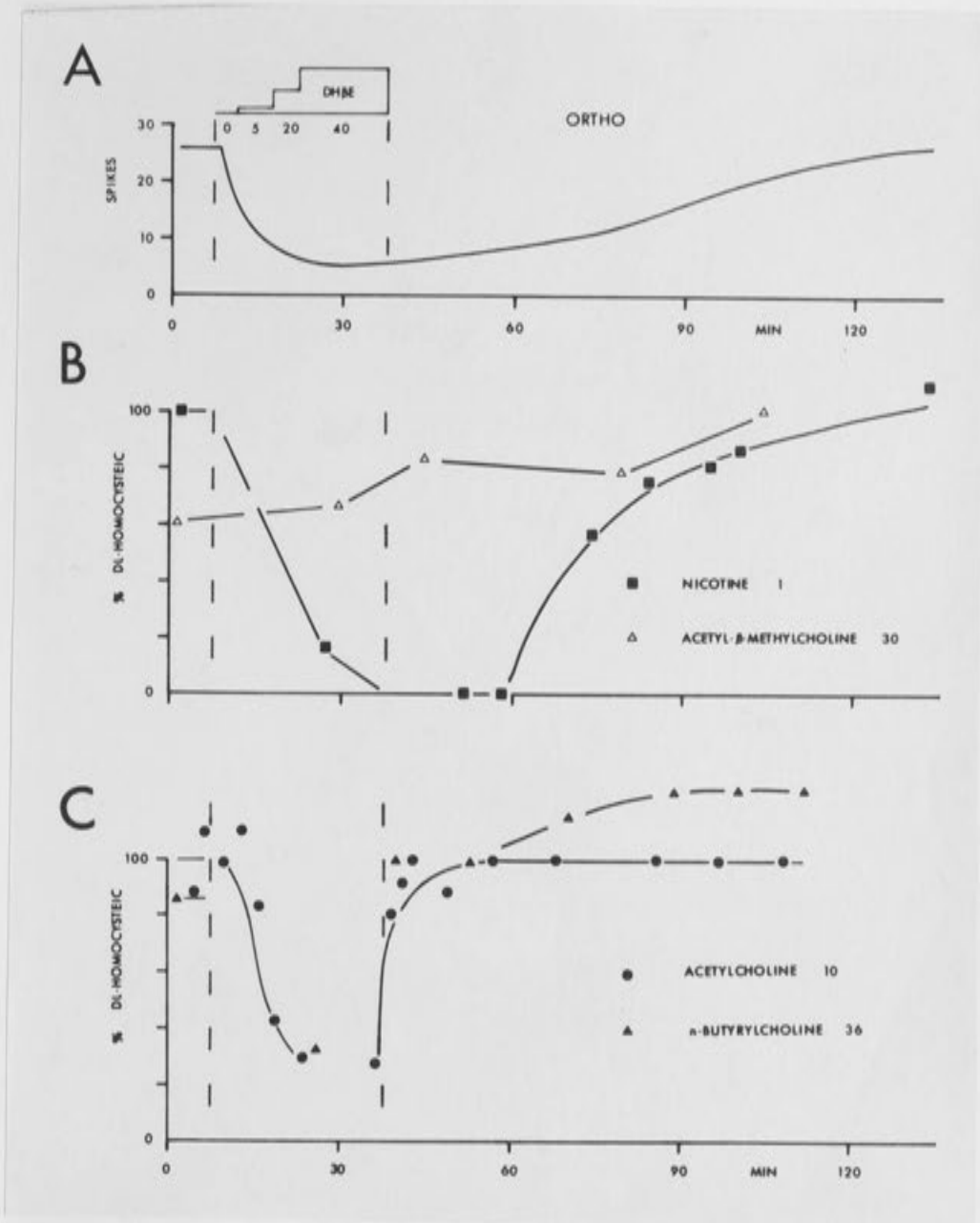

Fig. 38. A), Number of spikes (ordinate) in the initial response recorded from a Renshaw cell following a single ventral root volley: B), Frequency of firing produced by brief applications of nicotine (■) or acetyl-β-methylcholine (△), expressed as percentages of the control responses to DLH: C), Frequency of firing produced by brief applications of ACh (●) or n-butyrylcholine (▲) expressed as percentages of the control responses to DLH: Between the dotted lines, dihydro-β-erythroidine (10 mM in 165 NaCl) was ejected by electrophoretic currents increasing stepwise to 40 nA. Time, min.

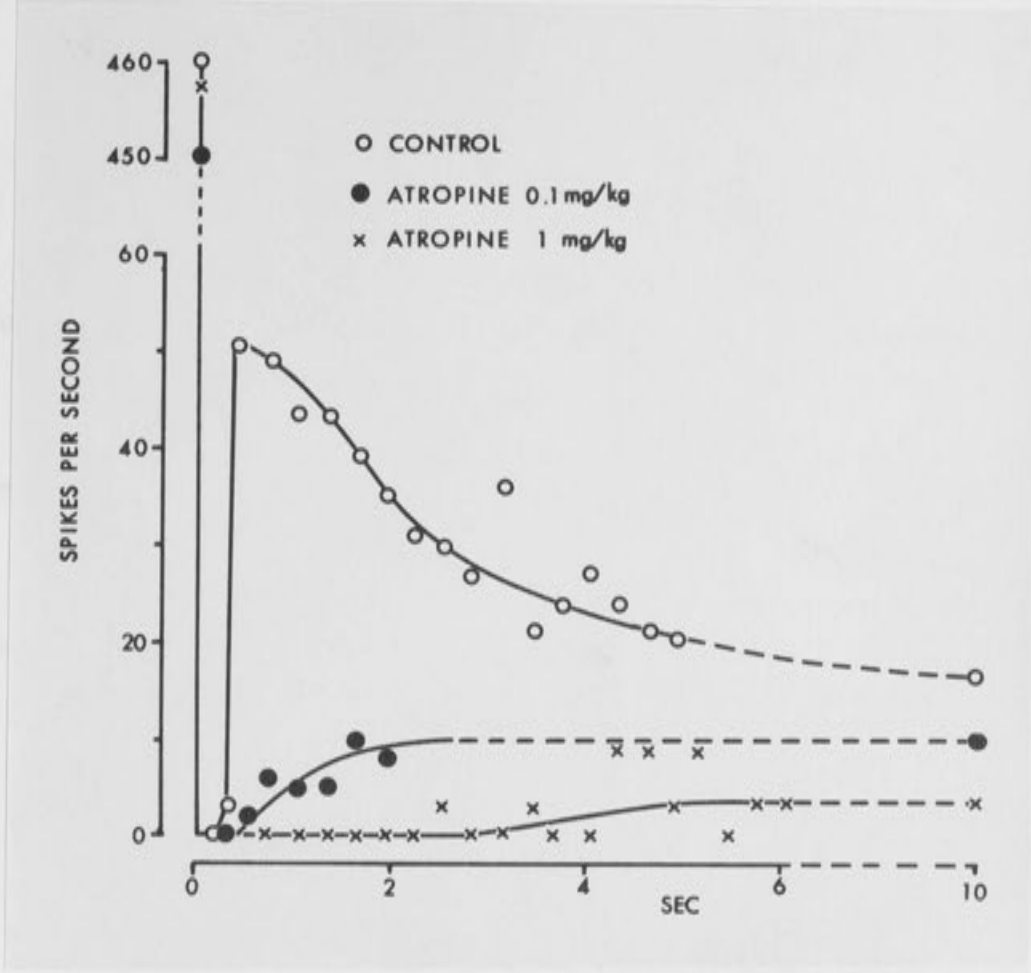

Fig. 39. Firing frequency (ordinate) of a Renshaw cell at various times following a single stimulus applied to the ventral root. The average frequency during the initial response is represented by the first point on the curves (note the break in the ordinate scale). Open circles, control response; closed circles, response obtained 8 min after intravenous injection of atropine sulphate (0.1 mg/kg); crosses, responses obtained 3 min after intravenous injection of atropine sulphate (1 mg/kg). The frequency shown at 10 sec is the average spontaneous firing rate. Time, sec. after stimulus.

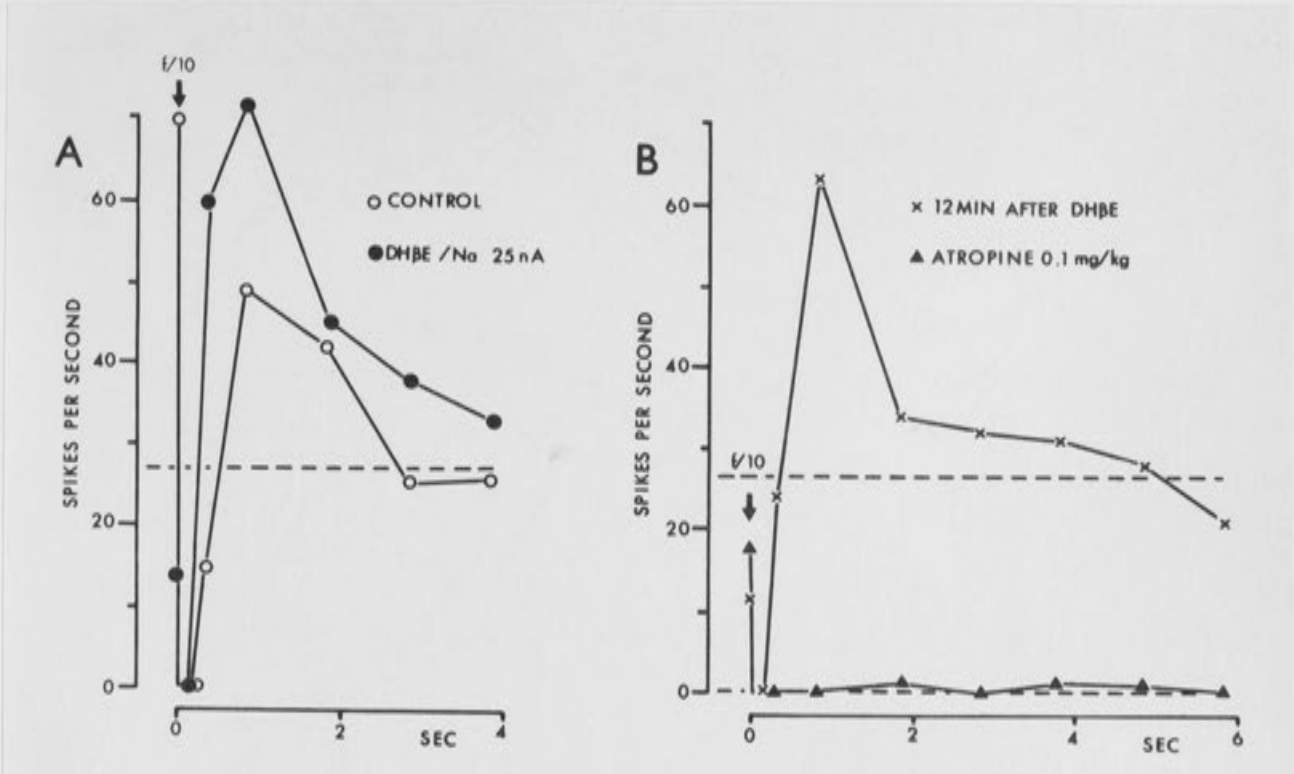

Fig. 40. As in Fig. 39, but average frequency during the initial response has been divided by 10 (f/10).

A) Open circles, control response; closed circles, response obtained during the electrophoretic application of dihydro-β-erythroidine (DHβE/Na) from a solution containing 20 mM DHβE in 165 mM NaCl.

B) Crosses, response obtained 12 min after termination of the ejection of DHβE; triangles, response obtained 3 min after the intravenous injection of atropine sulphate (0.1 mg/kg).

Horizontal dotted lines represent the average spontaneous firing rate. Time, sec after stimulus.

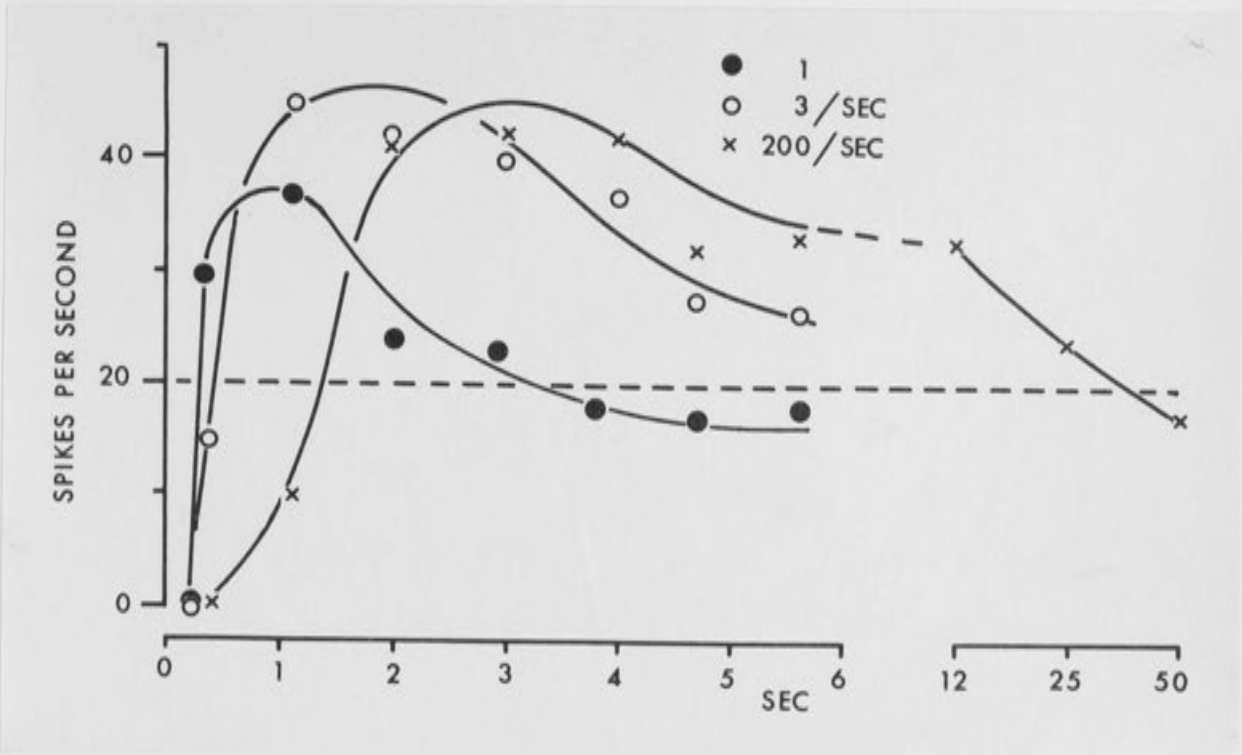

Fig. 41. Firing frequency (ordinate) of a Renshaw cell after a single shock to the ventral root (closed circles), following stimulation at 3/sec for 3 sec (open circles), and following stimulation at 200/sec for 3 sec (crosses). Abscissae, time (sec) after stimulus. The dotted line represents the average spontaneous firing frequency. Note that the initial response has been ommitted from the figure.

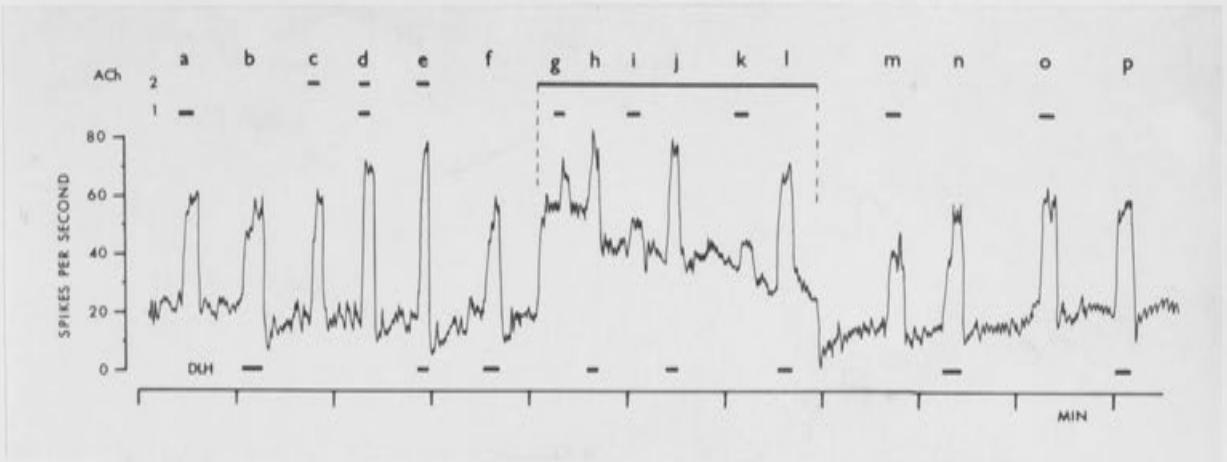

Fig. 42. Frequency of firing (ordinate) of a Renshaw cell plotted against time (abscissa). Two barrels of the micro-pipette contained 0.5 M ACh and a third barrel contained 0.2 M DLH. ACh was allowed to diffuse from either barrel 1 or barrel 2 (upper horizontal bars) by terminating the retaining current of 9nA. DLH was ejected by a current of 6 nA during the periods indicated by the lower horizontal bars. Time, min.

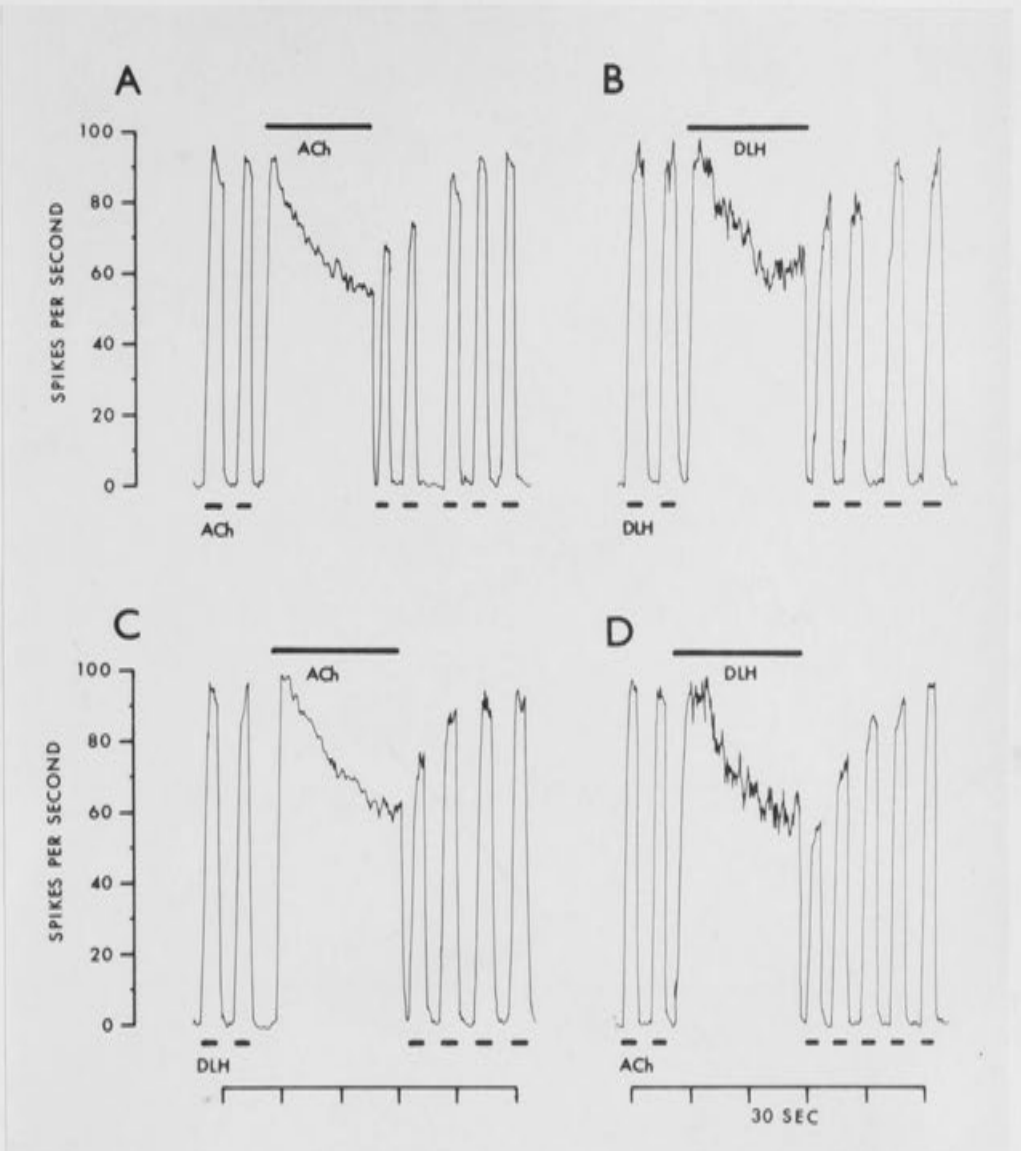

Fig. 43. Frequency of firing (ordinate) of a Renshaw cell plotted against time (abscissa). Effects of prolonged applications of ACh (A and C) or DL-homocysteic acid, DLH (B and D) on the firing rate produced by repeated brief (about 5 sec) current pulses of ACh (A and D) or DLH (B and C). Time, 30 sec.

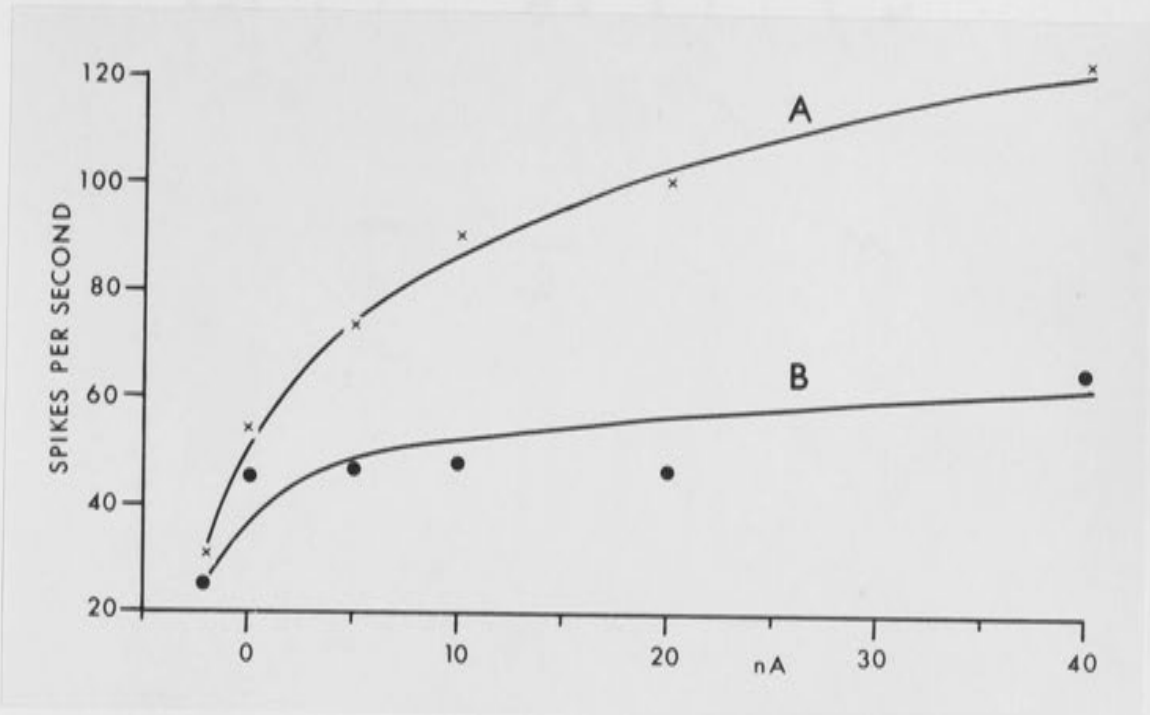

Fig. 44. Frequency of firing (ordinate) of a Renshaw cell, plotted against the current used to eject ACh (abscissa). The ACh was administered for periods of up to 2 min until the response had faded to an equilibrium value.

A) X - frequency of firing at the initial peak

B) ● - frequency of firing at equilibrium.

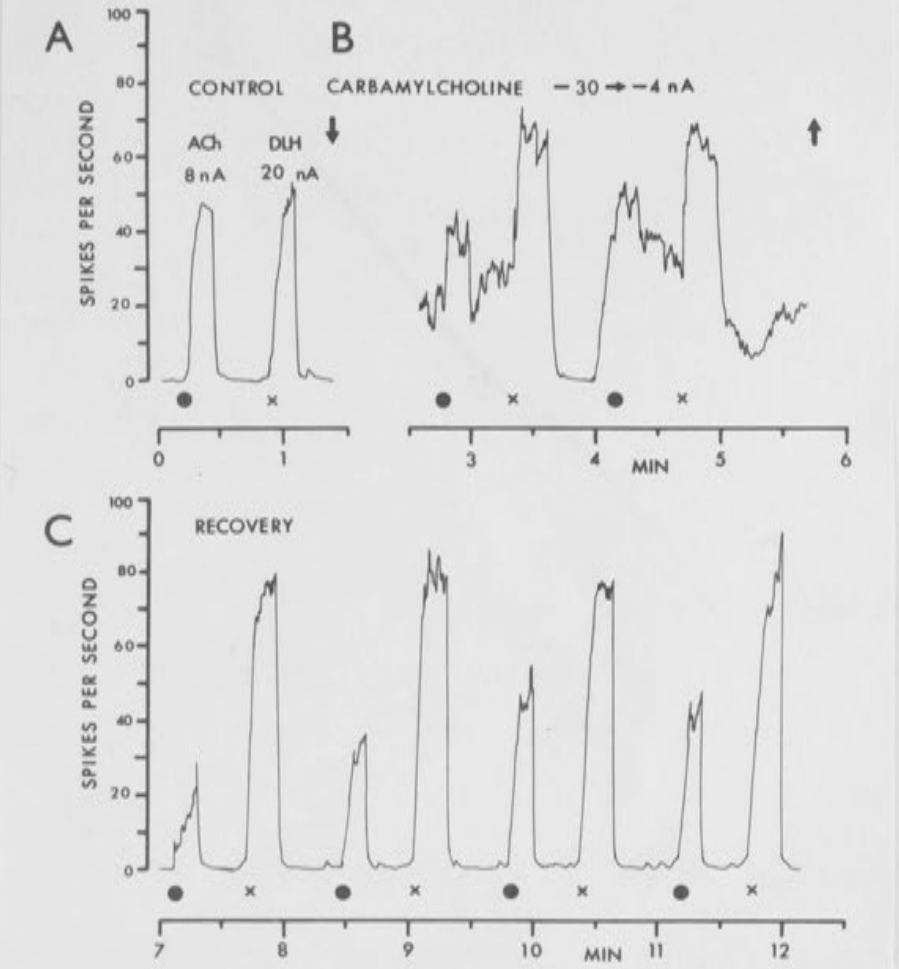

Fig. 45. Frequency of firing (ordinate) of a Renshaw cell plotted against time (abscissa). Responses were obtained to alternate ejections of ACh or DLH.

A; control responses to ACh (●) and DLH (X): in B, carbamylcholine was allowed to diffuse continuously from the micro-pipette by reducing the retaining current from -30 to -4 nA: C, recovery. Time, min.

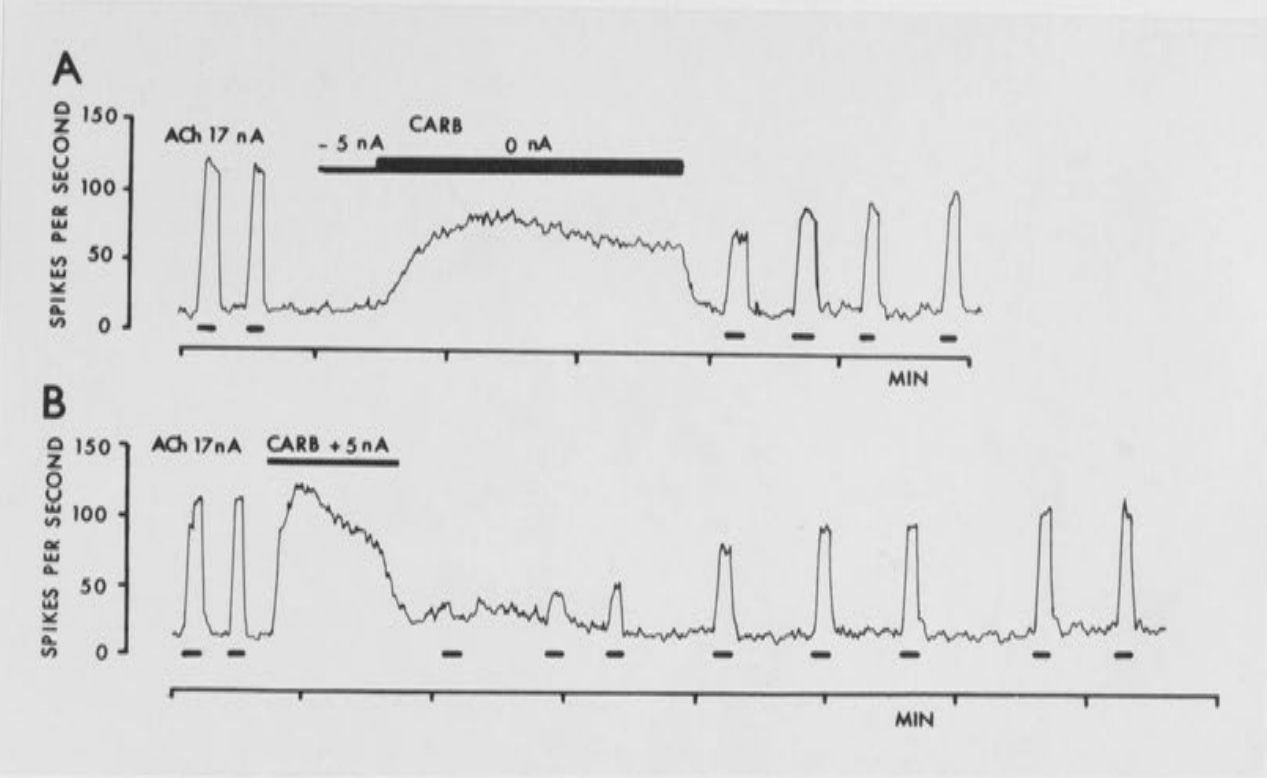

Fig. 46. Frequency of firing (ordinate) of a Renshaw cell, plotted against time (abscissa). During the periods indicated by the lower horizontal bars, ACh was ejected with an electrophoretic current of 17 nA. In A, carbamylcholine was allowed to diffuse from the electrode during the period indicated by the upper horizontal bar, first by reducing the retaining current from -8 nA to -5 nA, and then by turning off the retaining current (CARB 0 nA). In B, carbamylcholine was ejected by an electrophoretic current of 5 nA for the period indicated by the upper horizontal bar. Time, min.

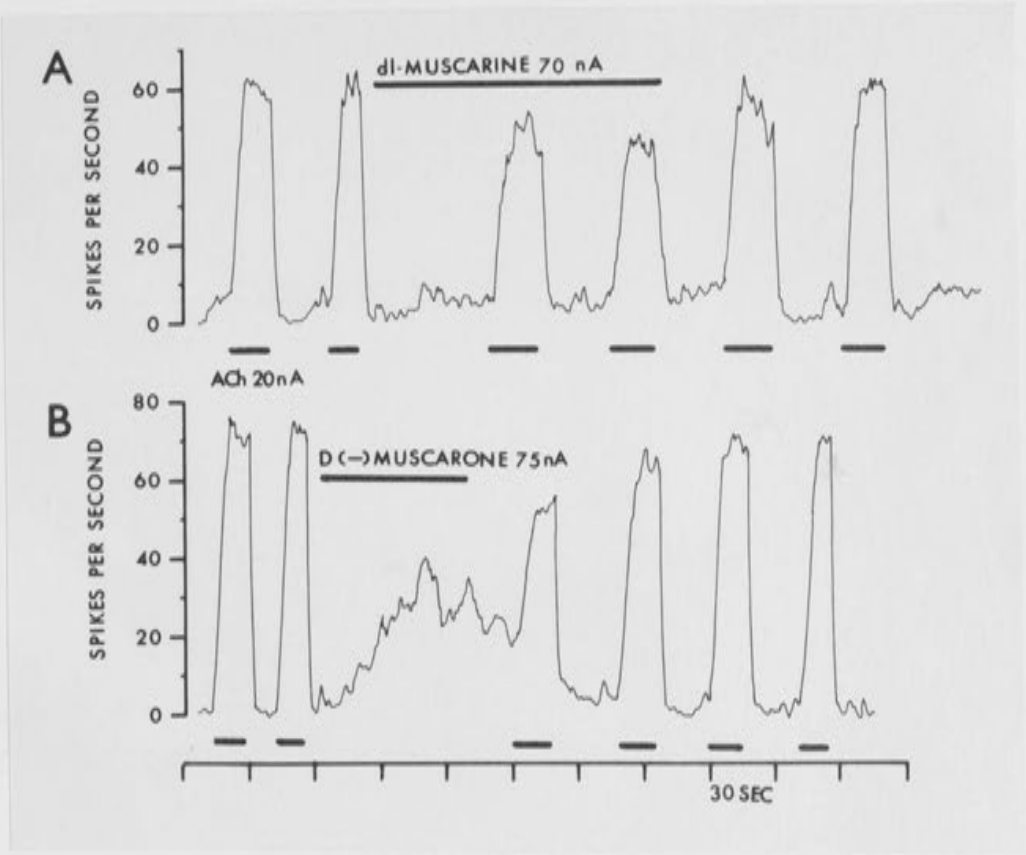

Fig. 47. Frequency of firing (ordinate) of a Renshaw cell, plotted against time (abscissa): A, effect of dl-muscarine (70 nA) on the firing produced by brief current pulses of ACh (20 nA) ejected during the periods indicated by the lower horizontal bars: B, effect of D(-) muscarone (75 nA) on the firing produced by brief current pulses of ACh: time, 30 sec.

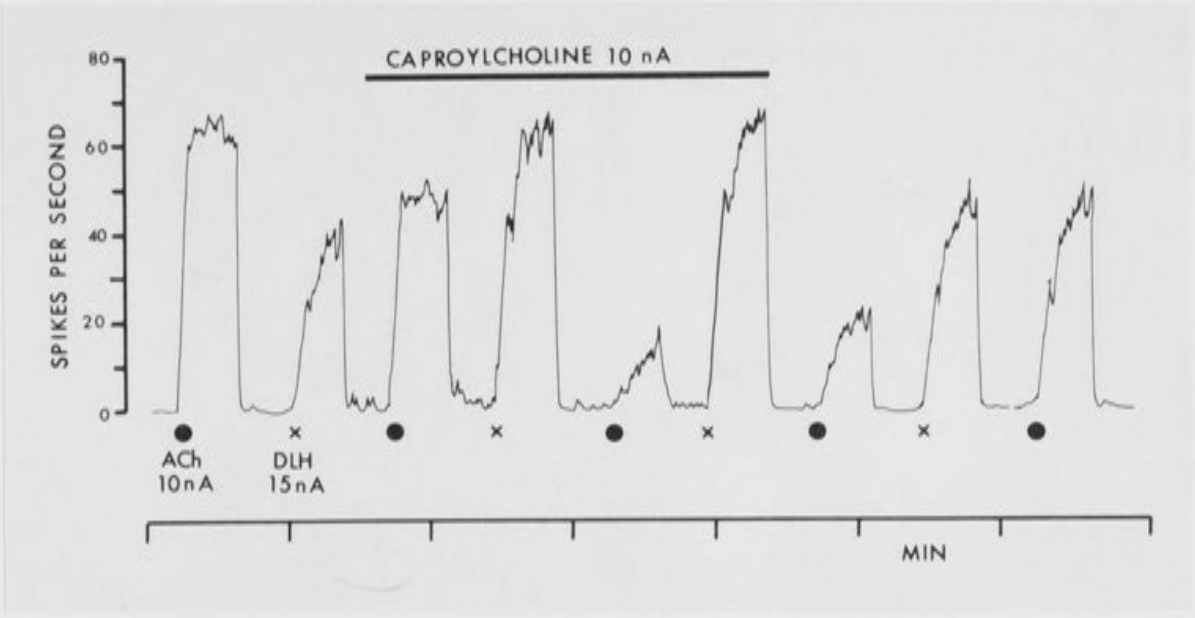

Fig. 48. Frequency of firing (ordinate) of a Renshaw cell, plotted against time (abscissa). Effect of caproylcholine on the firing induced by the electrophoretic application of ACh (●) or DLH (X), ejected alternately for periods of 25 sec. Time, min.

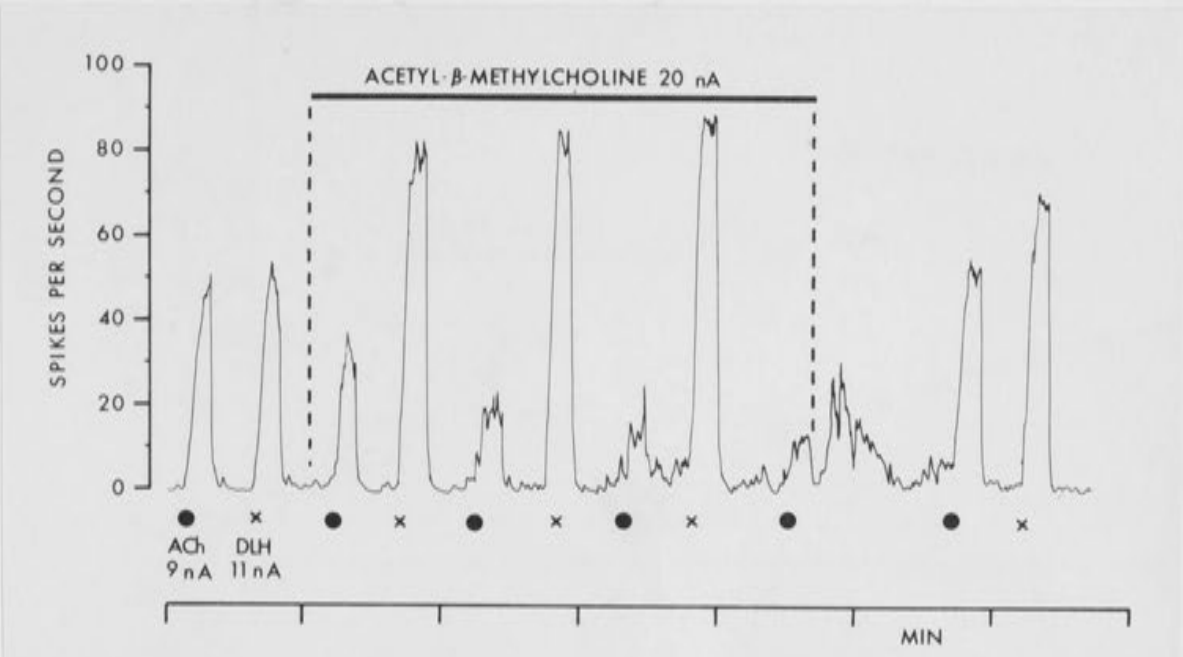

Fig. 49. Frequency of firing (ordinate) of a Renshaw cell, plotted against time (abscissa). Effect of a prolonged administration of acetyl-β-methylcholine (upper horizontal bar) on the firing produced by the alternate electrophoretic administration of ACh (●) or DLH (X) for periods of 12 sec. Time, min.

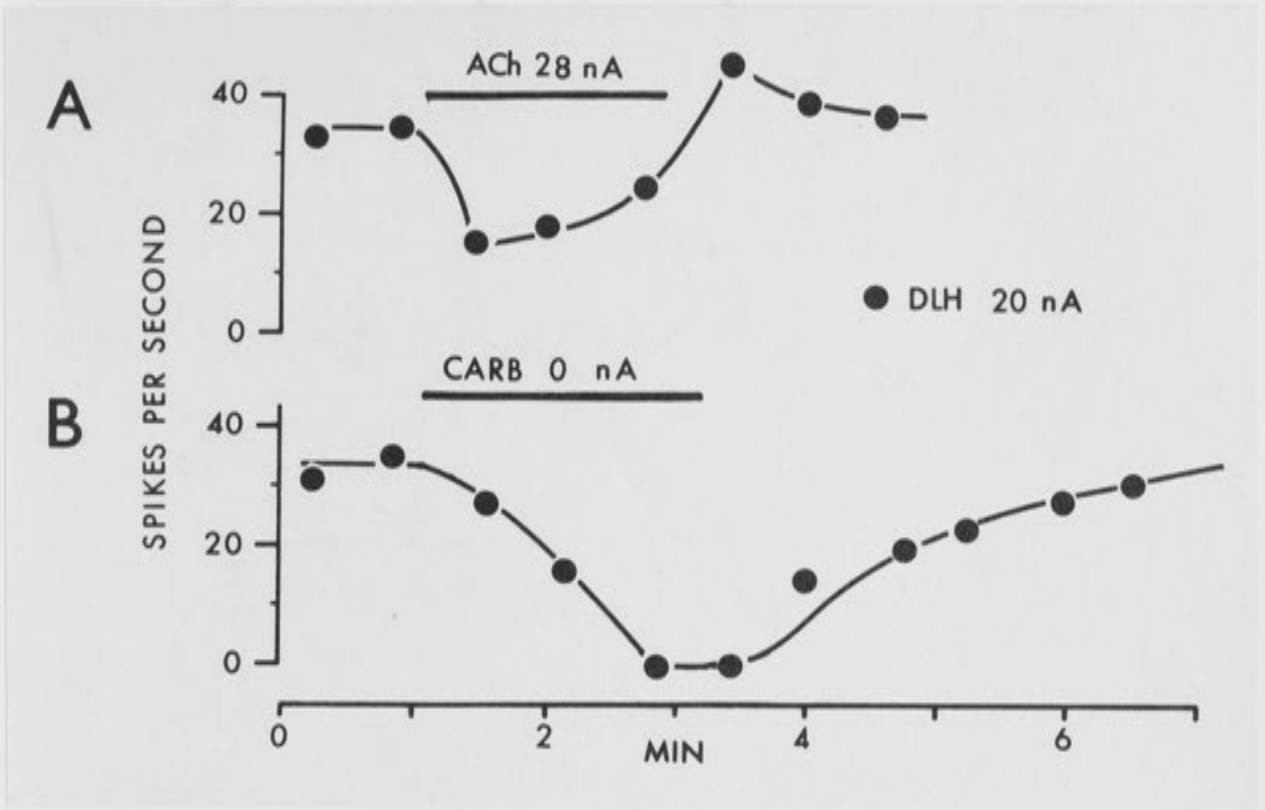

Fig. 50. Frequency of firing (ordinate) of a spinal interneurone, which responded to volleys in the sural nerve, plotted against time (abscissa). The points represent the maximum firing rate obtained during the ejection of brief (20 sec) current pulses of DLH (20 nA) repeated at intervals of 15 sec. A, effect of ACh continuously ejected during the period indicated by the upper horizontal bar: B, effect obtained when carbamylcholine was allowed to diffuse passively from the electrode (CARB 0 nA) by turning of the retaining current during the period indicated by the upper horizontal bar. The retaining currents were -18 nA for ACh and -25 nA for carbamylcholine. Time, min.

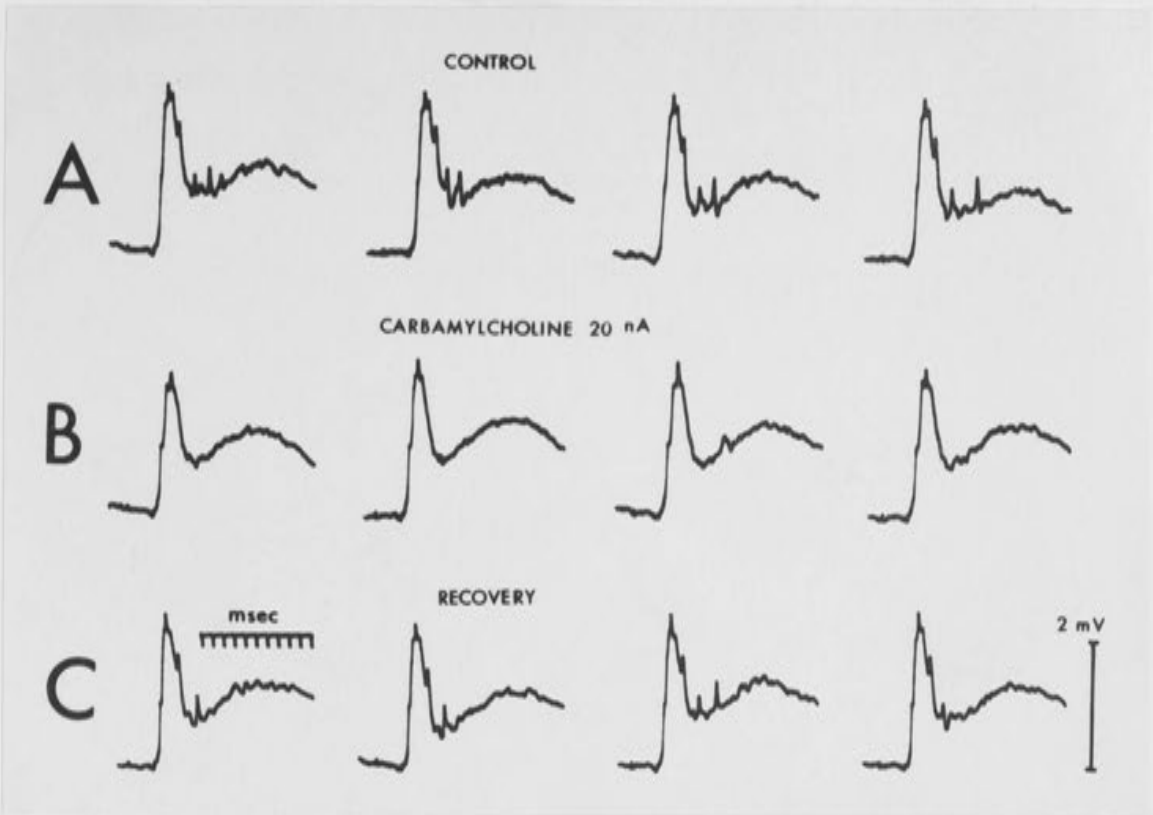

Fig. 51. Focal synaptic and spike potentials, recorded extracellularly from the same interneurone as in Fig. 50, following stimulation of the sural nerve.

A) Control records

B) Records obtained after carbamylcholine (20 nA) had been ejected from the micro-pipette for 1 min.

C) Records obtained 14 min after the termination of carbamylcholine administration. Calibrations, 2 mV and msec.

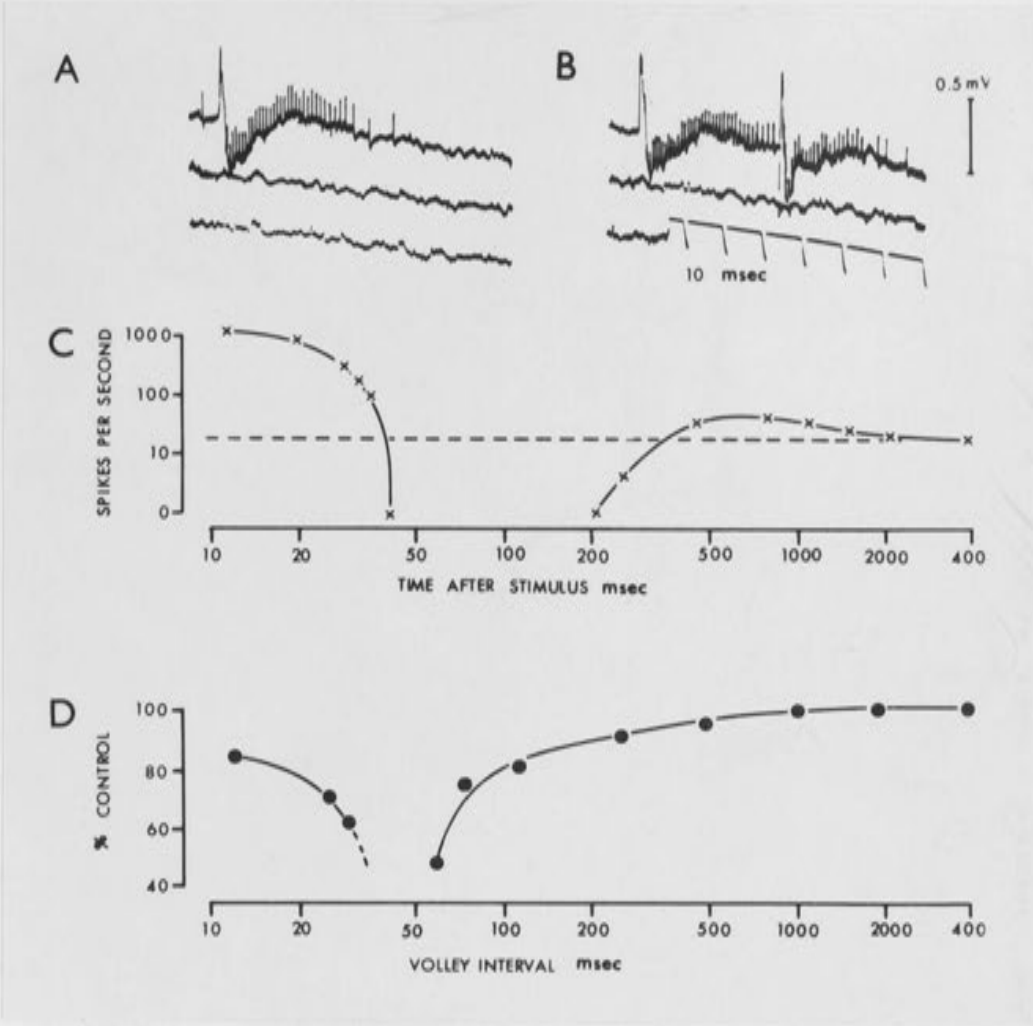

Fig. 52. A) Initial response recorded extra-cellularly from a Renshaw cell after a single supramaximal shock to the ventral root and B) after two volleys in the ventral root at an interval of 34 msec. C) Same cell; frequency of firing (ordinate), plotted against time (abscissae), after a single shock to the ventral root. Note the high frequency of firing during the initial discharge and the rapid decay, the pause and the late discharge. The dotted line represents the average spontaneous rate of firing. D) Number of spikes in the initial response following the second of two volleys, expressed as a percentage of the number of spikes following a single shock (ordinate), plotted against the time interval between volleys. Calibrations, 0.5 mV and 10 msec.

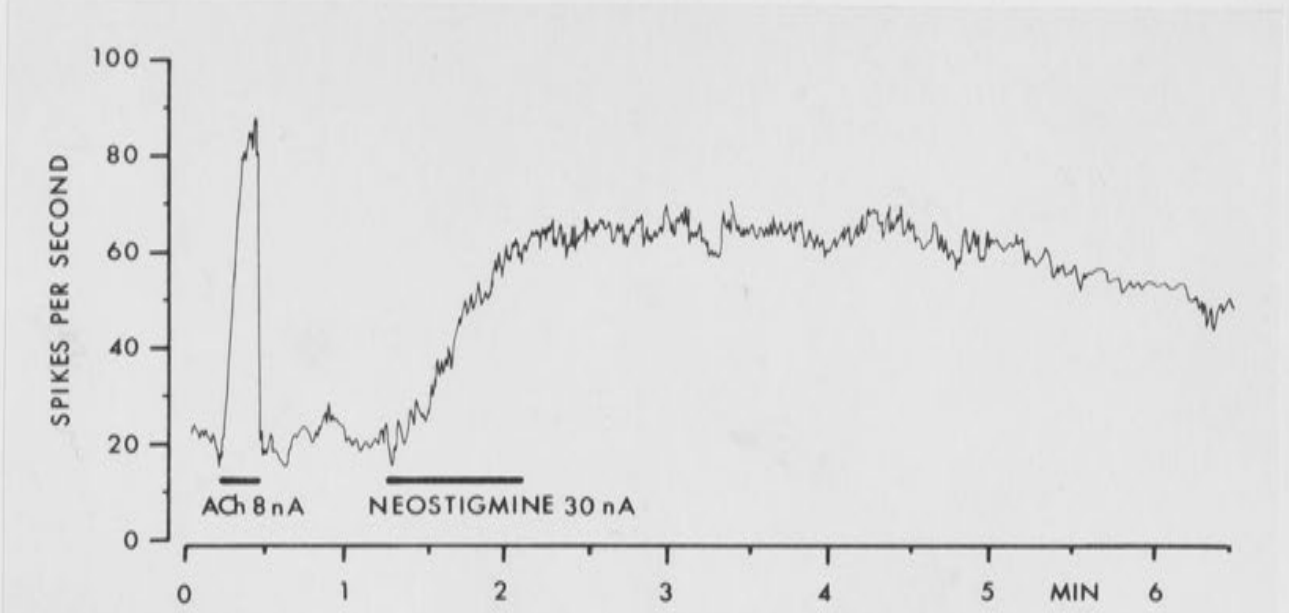

Fig. 53. Frequency of firing of a Renshaw cell (ordinate) plotted against time (abscissae). Response to a brief application of ACh and a more prolonged application of neostigmine from a solution containing 10 mM neostigmine in 165 mM NaCl. Time, min.

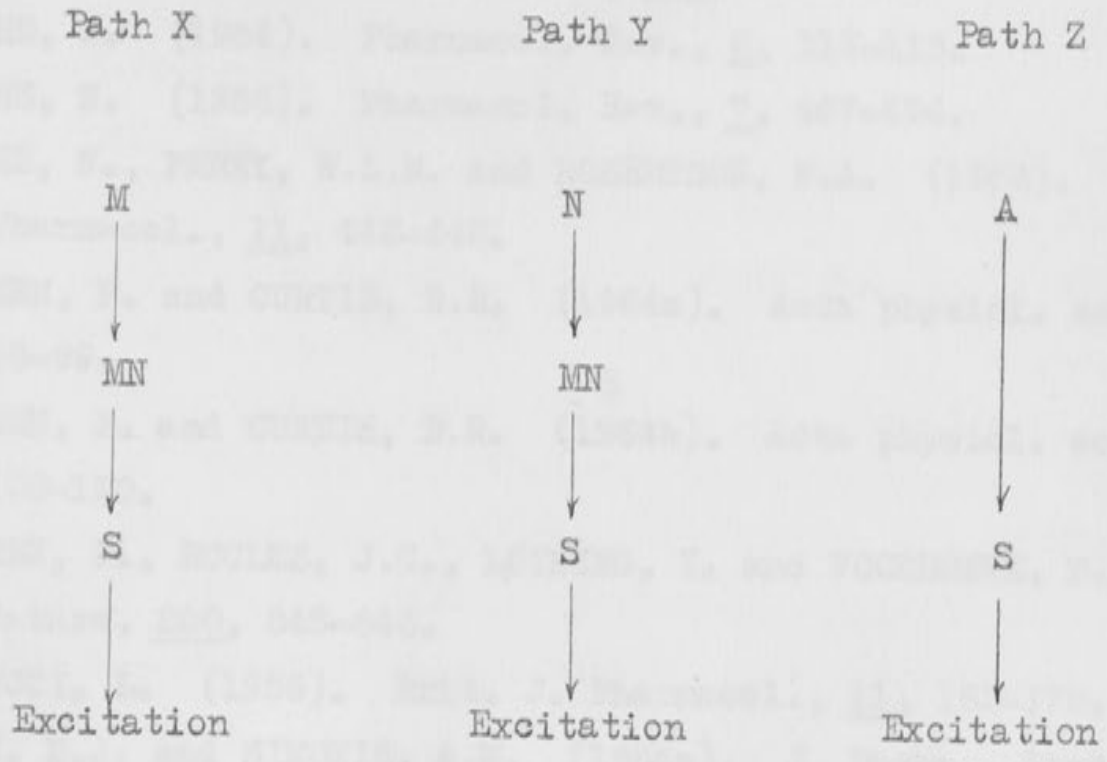

Fig. 54. Diagram depicting hypothetical pathways by which the process of receptor occupation may lead finally to excitation. M is a muscarinic receptor, N is a nicotinic receptor and A is an amino acid receptor. S is an intermediate receptor common to all pathways and MN is a receptor common only to paths X and Y. Drugs may interact with receptors to produce predominently excitation or block, depending upon the kinetics of their interactions.

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